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# Manipulation du pool d'hydrogène disponible dans le rumen pour limiter les émissions de méthane par les ruminants

# Manipulation of the hydrogen pool available in the rumen to reduce methane emissions from ruminants

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# Résumé

La réduction des émissions de méthane ($CH_4$) des ruminants permet de limiter les impacts environnementaux négatifs de leur élevage et d'améliorer leur efficacité digestive. Dans le rumen, le $CH_4$ est majoritairement produit par les méthanogènes à partir de l'hydrogène ($H_2$). La disponibilité de l'$H_2$ pour ces micro-organismes est réduite en limitant sa production par les protozoaires (*via* un apport de lipides ou extraits de plantes dans la ration) ou en stimulant des voies utilisatrices d'$H_2$ compétitives à la méthanogenèse (via un apport alimentaire de nitrate). Aucune étude n'a porté sur l'association de stratégies alimentaires jouant à la fois sur la production et l'utilisation d'$H_2$ pour diminuer les émissions de $CH_4$. Notre objectif était de comprendre l'importance des différentes voies métaboliques de l'$H_2$ dans le rumen. Nous avons émis l'hypothèse que manipuler simultanément la production et l'utilisation de l'$H_2$ permet une diminution plus importante des émissions de $CH_4$ plutôt que d'agir sur un seul niveau. Nos résultats expérimentaux ont montré l'additivité de l'association **lipides du lin-nitrate** sur la méthanogenèse des bovins. Cet effet était persistant mais non bénéfique pour les performances digestives et laitières des animaux. L'association **saponine de thé-nitrate** n'a pas été efficace pour réduire les émissions de $CH_4$ car l'effet dépressif de la saponine sur les protozoaires n'a pas été observé. Cette thèse ouvre la possibilité d'étudier le potentiel anti-méthanogène de nouvelles associations de stratégies alimentaires ayant des mécanismes d'action différents dans le rumen. Les conditions d'utilisation de ces stratégies en élevage devront être délimitées, et leur rentabilité prouvée, pour être acceptées par l'éleveur.

**Mots clés:** Fermentation; Hydrogène; Méthane; Microbiote; Ruminants; Stratégies de réduction.

# Abstract

Reduction of methane ($CH_4$) emissions from ruminants may limit the negative environmental impacts of their breeding and may improve their digestive efficiency. In the rumen, $CH_4$ is mainly produced by methanogens from hydrogen ($H_2$). Hydrogen availability for these micro-organisms is reduced by limiting its production by protozoa (*via* lipids or plants extracts supplementation in diets) or by stimulating pathways competing with methanogenesis for $H_2$ consumption (*via* nitrate supplementation in diets). No study tested association of dietary strategies acting on both $H_2$ production and consumption to reduce $CH_4$ emissions. Our objective was to understand the importance of the different $H_2$ metabolic pathways in the rumen. We assumed that simultaneous manipulation of $H_2$ production and consumption reduces $CH_4$ emissions to a higher extent than acting on a single pathway. Our experimental results showed the additive $CH_4$-mitigating effect of the association **lipids from linseed-nitrate** supplemented to bovine. This effect was persistent but not beneficial for digestive and lactating performances of animals. The association **tea saponin-nitrate** was not efficient to reduce $CH_4$ emissions, as the depressive effect of saponin towards protozoa has not been observed. This PhD thesis opens the possibility to study the anti-methanogenic potential of new association of dietary strategies having different mechanisms of action in the rumen. Conditions of use of these strategies at the breeding scale will have to be delineated, and their cost effectiveness proved to be accepted by farmers.



**Keywords:** Fermentation; Hydrogen; Methane; Microbiota; Mitigation strategy; Ruminants.

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# List of common abbreviations

AA: Amino acid

ADF: Acid detergent fiber

BCM: Bromochloromethane

BES: 2-bromo-ethane sulfonate

BW: Body weight

CP: Crude protein

CV: Coefficient of variation

DM: Dry matter

DMI: Dry matter intake

DNRA: Dissimilatory reduction of nitrate to ammonium

DOM: Digestible organic matter

$E_0$: Standard reduction potential

Fed: Ferredoxin

ΔG: Gibbs free energy

GE: Gross energy

GEI: Gross energy intake

GHG: Greenhouse gas

GWP: Global warming potential

Hb: Hemoglobin

LCA: Life cycle assessment

MetHb: Methemoglobin

NDF: Neutral detergent fiber

NPN: Non-protein nitrogen

OM: Organic matter

PUFA: Polyunsaturated fatty acid

RMSE: Residual mean square error

SD: Standard deviation

VFA: Volatile fatty acid

# General introduction

# I. CONSTRAINTS OF RUMINANTS BREEDING IN THE (FUTURE) AGRICULTURAL CHALLENGE: PRODUCE MORE AND BETTER WITH FEWER RESOURCES

Nowadays, the world population is significantly increasing, and is expected to pass from 7 billion (2014) to more than 9 billion in 2050 (Steinfeld et al., 2006). In addition, the individual level of consumption of animal products increased for the last 40 years in developing countries: between 1962 and 2003, meat and milk consumption passed respectively from 10 to 29 kg/person/year, and from 28 to 48 kg/person/year. Consequently, to fulfil the increasing demand of livestock products, a rise of meat and milk production is expected in the future (Figure 1), and development of sustainable systems of animal production that do not directly compete with mankind for foodstuffs is clearly necessary. **In this global context, ruminants play a major role in the human food supply chain by converting non-consumable fibrous feedstuff for humans to highly nutritional products. However, ruminants are criticized for their high contribution to greenhouse gas (GHG) emissions, and their impact on climate change is a major concern worldwide (Steinfeld et al., 2006).**

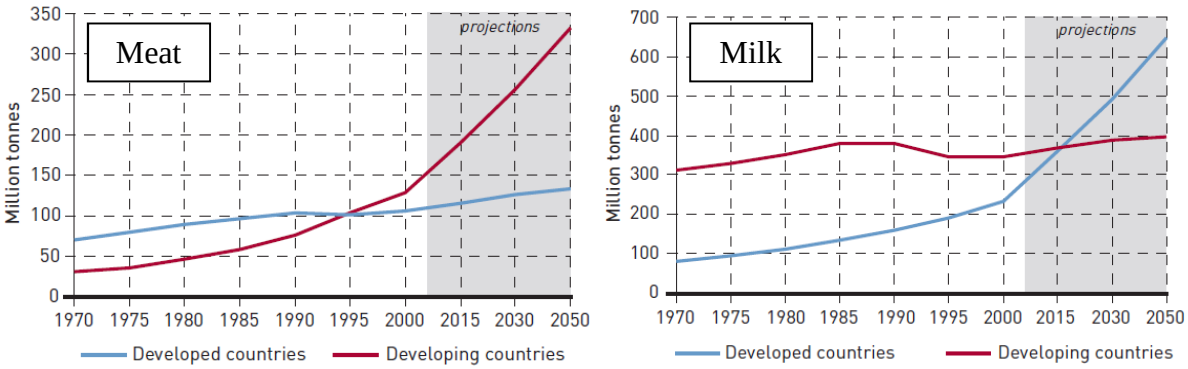


**Figure 1** Past and projected meat and milk production in developed and developing countries from 1970 to 2050 (from Steinfeld et al., 2006)

## *1.1. Interest of ruminants production: valorization of forage to highly nutritional products for human consumption*

Ruminants own a specific compartment at the beginning of their digestive tract, the rumen, in which feeds are fermented by microbes. This digestive particularity offers them the ability of producing human food using fibrous feedstuff that cannot be directly used by

humans and mono-gastric animals. On the contrary, pigs and chicken diets based on cereals are competitive with human food. However, among the future human protein sources, their feed conversion ratio (25 kg feed/kilogram edible weight) is the highest compared to pork (9.1), poultry (4.5) and crickets (2.1) (van Huis, 2013). Nowadays, ruminants are almost the sole source of milk for humans, by providing 644 million tons of milk (fat-protein corrected milk), among which dairy cattle is the main producer (Figure 2). Ruminants also provide 77.3 million tons of meat (carcass weight) representing 29% of the overall world meat production (Figure 2) (Gerber et al., 2013b).

Beside this major economic role, ruminants managed in extensive system also have a major role in terms of ecosystem services such as landscape management (Harrison et al., 2010). Among others, they help to maintain herbaceous areas difficult to access such as mountainous areas and prevent the development of weed and shrub species responsible for fire development and losses in plant biodiversity.

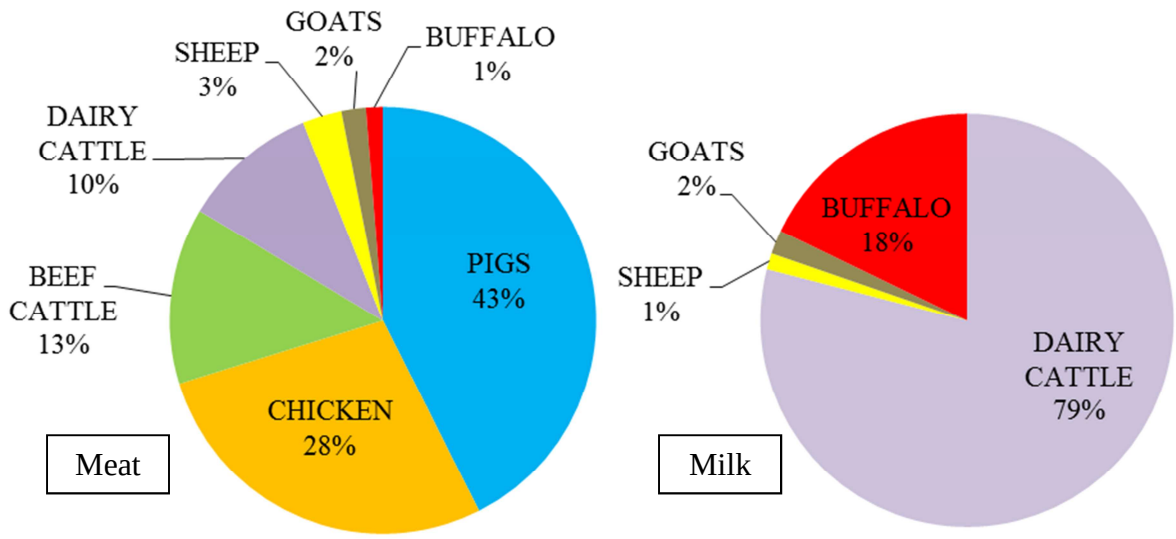


**Figure 2** Contribution of ruminants to the overall world meat and milk production (from Gerber et al., 2013b)

### *1.2. Downside of ruminants breeding: contribution to greenhouse gases emissions via enteric methane production*

Ruminants' production is accused of having a significant impact on the environment at the local and global level. Locally, the main issues concern intensive operations that contaminate the air, land or water with nitrogenous compounds and phosphorous releases. Globally, ruminants are pointed out for their contribution to GHG emissions, which occurs in

both intensive and extensive systems (Steinfeld et al., 2006). Carbon dioxide ($CO_2$), methane ($CH_4$) and nitrous oxide ($N_2O$) are the main GHG from anthropic origin (77, 14 and 8% of total GHG produced, respectively), with a global warming potential (GWP) of 1, 25 and 298 (IPCC, 2007). According to latest estimations (Gerber et al., 2013b), contribution of livestock supply chain to total anthropogenic GHG emissions raises at 14.5%, with $CH_4$, $N_2O$ and $CO_2$ emissions representing 44, 29 and 27%, respectively (expressed as $CO_2$-equivalent). Ruminants are mostly involved in $CH_4$ emissions, which represent 80% of $CH_4$ emissions from the livestock supply chain, the remaining 20% coming from manure management (Gill et al., 2010). In ruminants, 87% of $CH_4$ is produced in the rumen and eructated in the atmosphere, the remaining coming from the rest of the digestive tract (Murray et al., 1976). In France, cattle contributes more than 90% to total enteric $CH_4$ emissions (Figure 3; Vermorel et al., 2008).

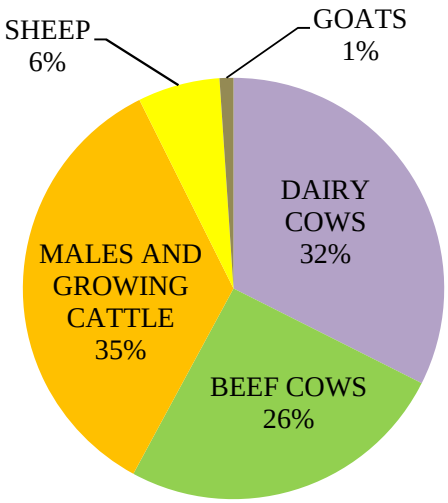


**Figure 3** Contribution of cattle, sheep and goat to total methane emissions from ruminants in France (from Vermorel et al., 2008)

In addition to be the main GHG emitted at the farm level, $CH_4$ released by ruminants constitutes an energetic loss for the animal, ranging from 2 to 12% of gross energy intake (GEI) by the animal (Johnson and Johnson, 1995) (*versus* 0.4% of digestible energy intake for pigs for instance; Noblet and van Milgen, 2004). Consequently, reduction of enteric $CH_4$ emissions from ruminants is desirable as a strategy to reduce global GHG emissions, without altering their productivity and their feed conversion efficiency.

Several strategies have been tested worldwide to limit methanogenesis (Grainger and Beauchemin, 2011; Gerber et al., 2013a; Knapp et al., 2014). Most of them consist in manipulating rumen parameters *via* feeding (modification of diet composition,

supplementation of dietary additives) or biotechnologies (defaunation, use of probiotics, exogenous microbial products or vaccines). Genetic selection of low $CH_4$-emitting animals is a more recent strategy. However, none of these strategies reduce $CH_4$ emissions on the long-term without losses in animals' performances, while being cheap and safe for the animal and the consumers. In this PhD thesis, we chose to work on dietary strategies as they allow getting results in a shorter term than other strategies.

## II. HOW TO REDUCE METHANE EMISSIONS FROM RUMINANTS VIA DIETARY STRATEGIES? OBJECTIVES AND SCIENTIFIC APPROACH OF THIS PHD THESIS

In the rumen, microbes find their energy in the form of ATP through dehydrogenation reactions releasing hydrogen ($H_2$). As soon as produced, $H_2$ is used by methanogenic archaea, a microbial group distinct from Eubacteria, to reduce $CO_2$ into $CH_4$ according to the following equation: $CO_2 + 4H_2 \rightarrow CH_4 + 2\ H_2O$. Methanogenesis is essential for an optimal performance of the rumen by avoiding $H_2$ accumulation which would inhibit fermentations. Consequently, $H_2$ and methanogenic archaea are the two determining parameters of $CH_4$ production in the rumen.

Recent results suggest that a reduction of methanogenic archaea activity can be achieved by a reduction of $H_2$ availability for these microorganisms (Popova, 2011). To reduce $H_2$ availability in the rumen, we classified the different dietary $CH_4$-mitigating strategies proposed by Gerber et al. (2013a) in two groups:

1/ Strategies reducing $H_2$ production (Table 1). This can be reached by limiting the number of protozoa in the rumen. Indeed, they are important $H_2$ producers and they would be involved in 10 to 35% of $CH_4$ production according to the diets (Morgavi et al., 2010). Addition of lipids or plants extracts (tannins, saponins, essential oils) in diets may reduce the number of protozoa in the rumen.

2/ Strategies stimulating $H_2$ consumption by other pathways (Table 2). Biochemical pathways using $H_2$ and/or chemicals directly inhibiting methanogenic archaea would allow reducing the proportion of $H_2$ directed towards methanogenesis. In this objective, diets including $H_2$-sinks (nitrate, sulfate), propionate enhancers (organic acids, high concentrate diets) or methanogens' inhibitors (chloroform) have been tested.

Today, a lot of these dietary strategies have been tested individually to reduce methanogenesis, but to our knowledge no studies reported the effects of the association of a strategy acting on $H_2$ production with a strategy acting on $H_2$ utilization.

**The objective of this PhD thesis was to better understand the importance of the different metabolic pathways of $H_2$ (production AND utilization) in the rumen, in order to propose and evaluate new dietary strategies to mitigate $CH_4$ emissions**. We assumed that manipulating at the same time production and utilization of $H_2$ allows a more important reduction of $CH_4$ emissions than acting on a single pathway (production OR utilization). To deal with this hypothesis, the scientific program of this PhD thesis was based on different approaches:

1/ Bibliographical approach. A literature review detailed the biological processes of $H_2$ production and consumption in the rumen. In addition, a quantitative analysis of the literature (meta-analysis) aimed at studying the influence of a variation of rumen protozoa concentration on $CH_4$ emissions.

2/ Experimental approach. We tested *in vivo* the $CH_4$-mitigating effect of different dietary strategies fed alone or in association to non-lactating and dairy cows. The originality of our approach consisted in combining strategies having different mechanisms of action on the rumen $H_2$ pool. Measurements of $CH_4$ emissions were linked with measurements of digestive efficiencies and animals' performances. When possible, rumen fermentations (fermentative and microbial parameters) were also analyzed in order to explain the mechanisms of action of tested strategies. In terms of rumen microbiota analysis, we mainly focused on populations producing (protozoa) and using (methanogens) $H_2$. To complete this *in vivo* approach, we estimated *in vitro* and in presence of different $H_2$-sinks, the distribution of $H_2$ in the fermentation end-products.

A final critical analysis of the overall results was made in the last section of this manuscript.

**Table 1** Overview of dietary enteric methane-mitigating strategies tested in ruminants to decrease hydrogen production (adapted from Gerber et al., 2013a)

| Active compound | $CH_4$-mitigating effect | Long term effect established | Risk for environment and animal | Effect on digestibility and animals' performances | Reference (Review or meta-analysis; Experimental studies) |
|---|---|---|---|---|---|
| Lipids | Significant effect of medium-chain (lauric, myristic acid) and polyunsaturated (linoleic and especially linolenic acid) fatty acids | Yes | No | Reduction of performances with doses higher than 4% added fat | Rasmussen and Harrison, 2011; Beauchemin et al., 2009; Machmüller et al., 2000; Martin et al., 2011; Martin et al., 2008 |
| Tannins | Variable effect according to tested source and dose | No | No | Frequent reduction of digestive efficiencies | Goel and Makkar, 2012; Animut et al., 2008; Grainger, 2009; Poungchompu et al., 2009 |
| Saponins | Variable effect according to tested source and dose | No | No | Variable effect according to tested source and dose | Holtshausen et al., 2009; Zhou et al., 2012 |
| Essential oils | Variable effect according to tested source and dose | No | No | Variable effect according to tested source and dose | Benchaar and Greathead, 2011; Calsamiglia et al., 2007; Klevenhusen et al., 2011; Shinkai et al., 2012 |

**Table 2** Overview of dietary enteric methane-mitigating strategies tested in ruminants to modify hydrogen consumption (adapted from Gerber et al., 2013a)

| Mechanism of action in the rumen | Active compound | $CH_4$-mitigating effect | Long term effect established | Risks for environment and animal | Effect on digestibility and animals' performances | Reference (Review or meta-analysis; Experimental studies) |
|---|---|---|---|---|---|---|
| Hydrogen-sinks | Nitrate | Significant and linear dose response effect | Yes | Risks of blood metHb; Nitrogen release poorly studied | No | Lee and Beauchemin, 2014b; El-Zaiat et al., 2014; Nolan et al., 2010; Van Zijderveld et al., 2011; Veneman et al., 2014 |
| | Sulfate | Significant effect | No | Risks of polioencephalomalacia | Not studied | Van Zijderveld et al., 2010 |
| | Nitroethane | Significant effect | No | Not studied | Not studied | Anderson et al., 2006; Brown et al., 2011 |
| Propionate enhancers | Malic acid, fumaric acid | Variable effect | No | No | No | Bayaru et al., 2001; Foley et al., 2009; Wood et al., 2009 |
| | Ionophores (monensin) | Variable effect. May also have a toxic effect towards protozoa | No | Not studied | No | Appuhamy et al., 2013; Guan, 2006; McGinn et al., 2004 |
| Methanogens inhibitors | Chloroform, BCM, BES, Cyclodextrin | Significant effect | No | Not sudied | No | Abecia et al., 2012; Knight et al., 2011; Mohammed et al., 2004 |
| | Fungal metabolites | Variable effect | No | Not studied | Not studied | Morgavi et al., 2013; Ramírez-Restrepo et al., 2014 |

BCM: bromochloromethane; BES: 2-bromo-ethane sulfonate; metHb: methemoglobin

# Literature review

# CHAPTER 1: Ruminal hydrogen production: importance of eukaryotes

In the rumen, dihydrogen (further named hydrogen or $H_2$) is produced by bacteria, protozoa and fungi during feed fermentation. This process is essential as it allows products reduced during feed fermentation (coenzymes and pyruvate) to be oxidized and used in further fermentative reactions.

Two oxidation-reduction[1] reactions are involved in $H_2$ production (Figure 4). In the first redox reaction (1: $Prod_{red} + 2H^+ + 2e^- + Fed_{ox} \rightarrow Prod_{ox} + Fed_{red} + 2H^+ + 2e^-$), the reduced product (Prod) is oxidized thanks to a ferredoxin (Fed). In the second redox reaction (2: $Fed_{red} + 2H^+ + 2e^- \leftrightarrow Fed_{ox} + H_2$), the reduced Fed is oxidized leading to $H_2$ synthesis.

The three following sections will describe i) the mechanisms of Fed reduction in prokaryotes and eukaryotes (first redox reaction in Figure 4), ii) the production of $H_2$ during the oxidation of Fed (second redox reaction in Figure 4), and iii) the solubility and concentration of $H_2$ in this digestive compartment.

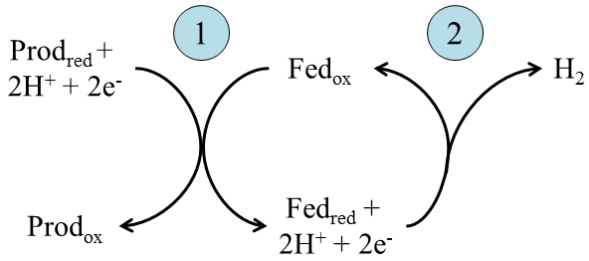


**Figure 4** Oxidation-reduction reactions involved in $H_2$ production ($Prod_{red}$ = reduced product, $Prod_{ox}$ = oxidized product, $Fed_{ox}$ = oxidized ferredoxin and $Fed_{red}$ = reduced ferredoxin) (from Hegarty and Gerdes, 1999)

## I. FERREDOXIN REDUCTION IN RUMEN MICROBES

### *1.1. Definition and microbial distribution of ferredoxin*

Ferredoxins are proteins able to shuttle electrons from a donor to an acceptor. This property is achieved thanks to the presence of an iron-sulfur cluster ($Fe_2S_2$ or $Fe_4S_4$) at the

[1] Oxidation-reduction reactions (or redox reactions) involve two redox couples exchanging electrons.

core of the protein. The redox state of the iron (Fe) atoms reflects the redox state of the Fed: when Fe is reduced ($Fe^{3+}$), the Fed is reduced and when Fe is oxidized ($Fe^{2+}$), the Fed is oxidized (Stiefel and George, 1994).

Ferredoxins have been reported in a wide range of bacteria from various biological environments (review of Yoch and Valentine, 1972). In the rumen, their presence have been reported in methanogenic archaea (Thauer et al., 1977), in several genera of bacteria such as *Ruminococcus*, *Selenomonas*, *Megasphaera* and *Desulfovibrio* (Glass et al., 1977; Michel and Macy, 1990; Valentine and Wolfe, 1963), in the entodiniomorphid and holotrich orders of protozoa (Paul et al., 1990; Yarlett et al., 1985) and in the anaerobic fungus *Neocallismatix* spp. (Rees et al., 1998; Yarlett et al., 1986).

### *1.2. Ferredoxin production during microbial feed fermentation*

Reduced Fed are produced during feed fermentation. As carbohydrates are the predominant components in ruminants' diet, Fed are mostly reduced during the fermentation of sugars into volatile fatty acids (VFA). To a minor extent, Fed are also reduced during protein fermentation (Czerkawski, 1986).

#### 1.2.1. Production of reduced ferredoxins during carbohydrates fermentation

When carbohydrates enter the rumen, they are hydrolyzed by several microbial exogenous enzymes which act in synergy to generate glucose or xylulose. The subsequent fermentation of these two products leads to VFA, which are the main source of energy for the ruminant. In prokaryote, the fermentation of glucose mainly generates acetate, butyrate and propionate, whereas in eukaryote, acetate and butyrate are mainly synthesized (Jarrige et al., 1995; Williams and Coleman, 1997).

The production of reduced Fed during glucose fermentation is different between VFA. In prokaryotes (bacteria; Figure 5), the production of two moles acetate or one mole butyrate from glucose generates 8 and 4 moles reduced Fed. The production of propionate requires 4 moles reduced Fed. In eukaryotes (protozoa and fungi; Figure 6), the production of two moles acetate from one mole glucose leads to the production of 8 or 12 moles reduced Fed, depending on the fermentative route (through malate). The formation of one mole butyrate generates 4 moles reduced Fed. Finally, knowing that the ratio of acetate to propionate to butyrate to valerate is approximately 66:19:11:4 in the rumen (Sauvant et al., 2011), it is clear that glucose fermentation to VFA results in an important production of reduced Fed.

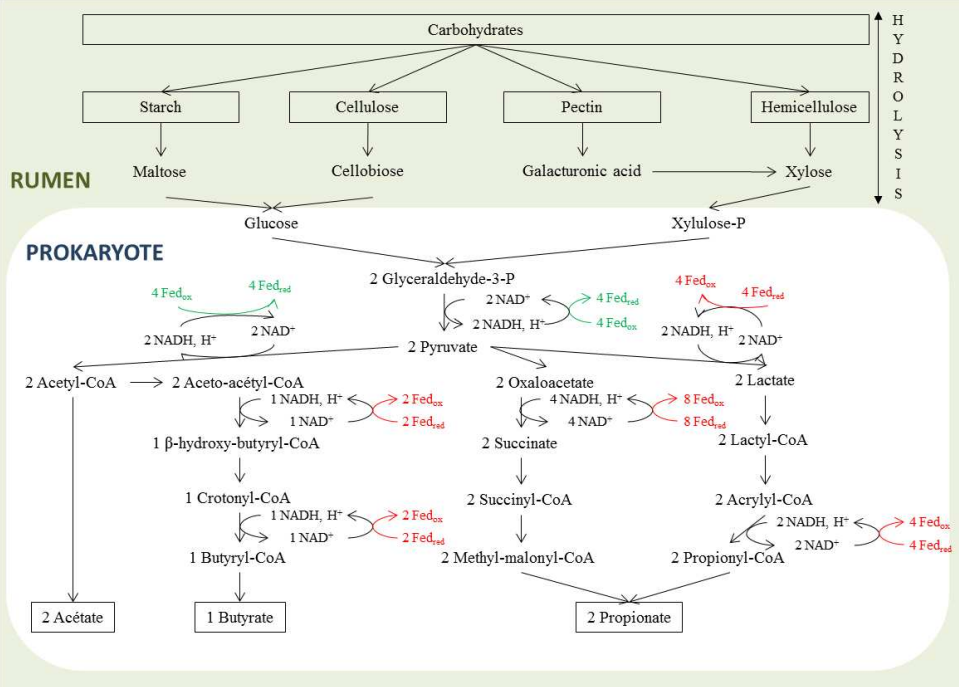


**Figure 5** Carbohydrates hydrolysis and glucose fermentation pathway in prokaryotes. Reactions leading to the production of reduced ferredoxins are in green. Reactions leading to the production of oxidized ferredoxins are in red. (from Fonty et al., 1995; Prescott et al., 2010)

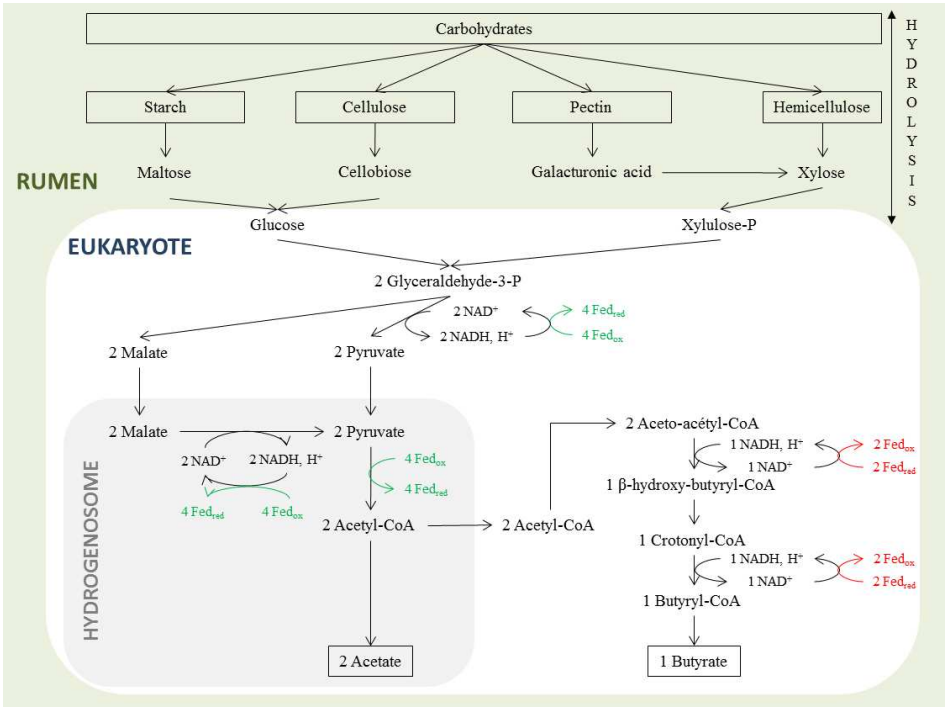


**Figure 6** Carbohydrates hydrolysis and glucose fermentation pathway in eukaryotes including hydrogenosome. Reactions leading to the production of reduced ferredoxins are in green. Reactions leading to the production of oxidized ferredoxins are in red. (from Müller, 1993; Williams and Coleman, 1997)

1.2.2. Production of reduced ferredoxins during protein fermentation

Proteins entering the rumen are hydrolyzed by exogenous enzymes to generate amino acids (AA). During further microbial fermentation of AA, Fed are also reduced (Wu, 2013; Figure 7).

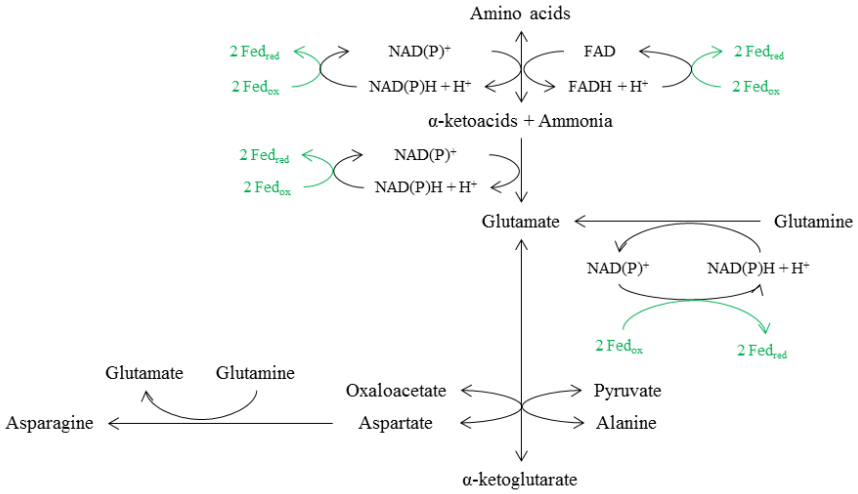


**Figure 7** Microbial fermentation of amino acids in the rumen: exemple of glutamine and asparagine, which serve as substrates for the microbial synthesis of all other amino acids. Reactions leading to the production of reduced ferredoxins are in green. (from Wu, 2013)

## *1.3. Mechanisms of ferredoxin reduction*

Ferredoxins are reduced during the oxidation of coenzymes in both prokaryotes and eukaryotes, but also during pyruvate oxidation in eukaryotes only (Figure 5, Figure 6).

1.3.1. Ferredoxin reduction from coenzymes

Ferredoxin reduction from coenzymes[2] takes place in the cytoplasmic membrane of rumen prokaryotes and in the cytosol or in the hydrogenosome of eukaryotes (more details about hydrogenosome will be given in following sections). The reaction is carried out by a coenzyme dehydrogenase which uptakes the electrons from coenzymes reduced during feed fermentation to the oxidized Fed (Valentine and Wolfe, 1963). The reaction catalyzed by the NAD(P)H dehydrogenase is:

[2] Coenzymes are organic compounds which include non-vitamin and vitamin derivatives. Adenosine triphosphate (ATP) responsible for phosphate transfer is an example of non-vitamin derivative. Vitamin derivatives include nicotinamide adenine dinucleotide ($NAD^+$) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) derivating from vitamin $B_3$ (niacin) or flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) derivating from vitamin $B_2$ (riboflavin). These coenzymes serve as reversible carriers of reducing equivalents. (Broderick, J. B. 2001. Coenzymes and cofactors. Encyclopedia of life sciences, Nature Publishing Group.)

$$NAD(P)H + H^+ + 2\ Fed_{ox} \rightarrow NAD(P)^+ + 2\ Fed_{red} + 2H^+ + 2e^-$$

And the reaction catalyzed by the FADH dehydrogenase is:

$$FADH + H^+ + 2\ Fed_{ox} \rightarrow FAD + 2\ Fed_{red} + 2H^+ + 2e^-$$

Where $NAD(P)H + H^+$ and $FADH + H^+$ are the reduced coenzymes, $Fed_{ox}$ is the oxidized Fed, $NAD^+$ and FAD are the oxidized coenzymes, $Fed_{red}$ is the reduced Fed and $e^-$ is the electron. As the standard reduction potential of NAD and FAD are more positive than the one of Fed (more precisions about thermodynamic laws are given in chapter 2), the NAD(P)H and FADH dehydrogenases can work only in the direction of Fed reduction, and the reverse direction is strongly inhibited by NAD(P)H, $H^+$ or FADH, $H^+$ (Gottschalk, 1986).

Whereas eukaryotes and some bacteria such as *Ruminococcus albus* do not require additional electron carriers (Glass et al., 1977), some bacteria require a cytochrome (cytochrome c) which is an intermediate electron carrier between the coenzyme and the Fed (Dolla et al., 1990). This transport of electrons through different electron carriers is named the electron transport chain. The presence of cytochrome b, a sub-unit of cytochrome c, has been detected in different rumen bacterial species such as *Prevotella* (White et al., 1962), *Fibrobacter succinogenes* (Reddy and Bryant, 1977), *Selenomonas ruminantium* (Stewart et al., 1997) and *Wolinella succinogenes* (Kern and Simon, 2009; Kröger et al., 2002).

Then, Fed reduction allows re-generating coenzymes into their oxidized form. As the concentration of coenzymes is fixed in the rumen, this process is essential to let the fermentations going on (Hegarty and Gerdes, 1999). To our knowledge, very few information exists about the concentration of coenzymes in the rumen. Indeed, coenzymes concentration is difficult to measure as they are quickly metabolized and their dosage requires an extraction from the cell followed by purification. In an *in vivo* experiment, the concentration of NAD analyzed from cells pellets from the ruminal fluid of dairy cows fed a barley or an oat based diet averaged 3.21 and 2.29 µ*M*, respectively (Abdouli and Schaefer, 1986).

### 1.3.2. Direct ferredoxin reduction from pyruvate

In rumen eukaryotes, Fed reduction also occurred during the direct oxidation of pyruvate. This process occurs in the cytosol but may also occur within a specific organelle called the hydrogenosome (Martin and Müller, 2007; Müller et al., 2012).

*Structure and occurrence of hydrogenosomes.* Hydrogenosomes are membrane-bound organelles (Figure 8) which have only been reported in several anaerobic or microaerophilic unicellular eukaryotes. They share some similarities with mitochondria as they both use

pyruvate as a major substrate leading to the production of acetyl-CoA and ATP (Müller, 1993). However, as they do not co-exist with mitochondria, it was hypothesized that these two organelles come from the same symbiont which would have evolved differently according to its environment. In aerobic environment, this symbiont would have generated the mitochondria and in anaerobic environment, it would have created the hydrogenosome. Genomes comparison validated this assumption, as hydrogenosomal genome appeared to be highly related to mitochondrial genome (Akhmanova et al., 1998; Martin, 2005).

Hydrogenosomes have been reported in several rumen protozoa: *Polyplastron multivesiculatum* (Paul et al., 1990), *Eudiplodinium maggi* and *Epidinium ecaudatum* (Yarlett et al., 1984), *Dasytricha ruminantium* (Yarlett et al., 1981), *Isotricha prostoma* and *Isotricha intestinalis* (Yarlett et al., 1983). This organelle has also been reported in some rumen fungi such as *Neocallimastix patriciarum* (Yarlett et al., 1986). Nevertheless, hydrogenosomes have not been detected in some protozoal species such as *Entodinium caudatum*, *Entodinium simplex* and *Diploplastron affine* (Yarlett et al., 1984). On the contrary, they host a mitosome, a recently discovered organelle which does not produce energy, and whose function has not been clarified (Hackstein, 2010). For these species, $H_2$ production and associated mechanisms take place in the cytosol of the cell.

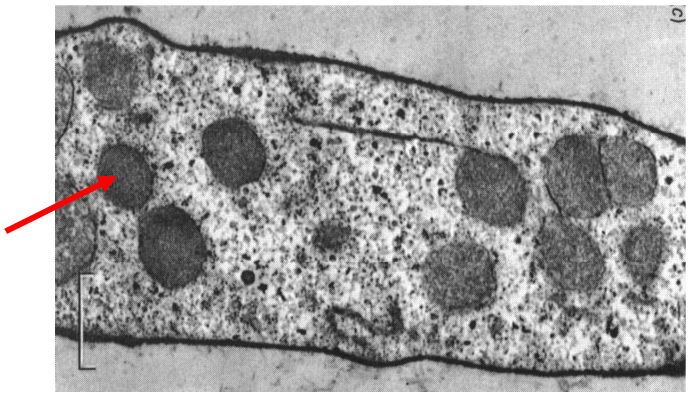

**Figure 8** Electron micrograph of rumen fungus (*Neocallimastix patriciarum*) showing hydrogenosome organelles. The scale bar represents 1 µm, the red arrow points out one hydrogenosome. (from Yarlett et al., 1986)

*Mechanism of pyruvate oxidation.* Eukaryotes directly reduce pyruvate to acetyl-CoA, which is further converted to acetate or butyrate (Yarlett et al., 1985). The conversion of one mole pyruvate to one mole acetyl-CoA is performed by a pyruvate-ferredoxin oxidoreductase through the reduction of two moles Fed:

$$\text{Pyruvate} + 2\ \text{Fed}_{ox} \rightarrow \text{Acetyl-CoA} + 2\ \text{Fed}_{red}$$

This reaction is direct as no electron carriers such as cytochromes or coenzymes are required between the pyruvate and the Fed (Müller et al., 2012).

## II. HYDROGEN PRODUCTION DURING FERREDOXIN OXIDATION

To ensure continuous fermentations, reduced Fed need to be oxidized. This process is concomitant to the production of $H_2$ by a hydrogenase. Hydrogenases are present in a large number of prokaryotes (Schwarz and Friedrich, 2003) and eukaryotes (Müller et al., 2012). They are responsible for the reduction or oxidation of $H_2$:

$$2H^+ + 2e^- \leftrightarrow H_2$$

The direction of this reversible reaction depends on the redox potential of the environment (Vignais and Colbeau, 2004). The rumen being a highly reducing environment (Eh = -150 to -400 mV; Marden, 2007), the reaction is directed towards $H_2$ production.

### *2.1.General composition and classification of hydrogenases in anaerobic environments*

Most hydrogenases are metallo-enzymes. Their catalytic site consists of a heterodimer, which is a protein complex made of two different sub-units. The first sub-unit is the iron-sulfur cluster [$Fe_2$-$S_2$, $Fe_3$-$S_4$ or $Fe_4S_4$] which is responsible for the transport of electrons to the second sub-unit, or active site (Beinert et al., 1997). Hydrogenases can be sorted into three classes according to the metal atoms of their active site (general reviews about hydrogenases: Vignais et al., 2001; Vignais and Colbeau, 2004):

- <u>The [Ni-Fe] hydrogenases</u> are the most numerous ones and are found in both bacteria and archaea. They are divided into four groups. The first group gathers respiratory hydrogenases which are responsible for $H_2$ oxidation coupled to the reduction of electron acceptors ($NO_3^-$, $SO_4^{2-}$, $CO_2$, $O_2$…). Hydrogenases of the second group are responsible for the activation of the expression of hydrogenase structural genes (Barz et al., 2010). The third group of hydrogenases is associated to the coenzymes dehydrogenase in charge of the reduction of $H_2$ and the oxidation of reduced cofactors (NAD(P)H, $H^+$). The last and fourth group of hydrogenases is mostly involved in the disposal of reducing equivalents produced during carbon monoxide or formate oxidation.

- The [Fe-Fe] hydrogenases active site consists of a [Fe-Fe] subunit, also called H-cluster. These hydrogenases are found in anaerobic prokaryotes, but they also are the only type of hydrogenases found in eukaryotes such as protozoa or fungi. In these microorganisms, they are exclusively located in the hydrogenosomes. These enzymes are mostly involved in $H_2$ production. Due to their occurrence in very diverse microbes, they can be associated to various electron acceptors and donors.

- The [Fe-S] cluster free hydrogenases are found in some specific methanogenic archaea. These enzymes do not contain nickel as they mostly grow under nickel limited environment. They also differ from the [Ni-Fe] and [Fe-Fe] hydrogenases by their primary and tertiary structures and, by the fact that, iron is not redox active. Consequently, they have specific cofactors and they do not catalyze the oxidation or the reduction of $H_2$. On the contrary, they are mostly involved in the reduction of methylene groups.

### *2.2. Hydrogenases involved in ruminal hydrogen production*

The rumen anaerobic environment offers good conditions for the production and activity of hydrogenases, as oxygen ($O_2$) negatively affects most of hydrogenases activity (La Penna, 2010; Stripp et al., 2009). Indeed, $O_2$ would react with the active site of the enzyme, creating a superoxide ($E_0$ = +0.9V) which may be released only in the presence of an electron acceptor with a higher standard reduction potential. More detailed thermodynamics approach will be given in Chapter 2.

Hydrogenases have been purified and detected in several rumen bacterial species such as *Bacteroides clostridiiformis*, *Butyrivibrio fibrisolvens*, *Eubacterium limosum*, *Fusobacterium necrophorum*, *Megasphaera elsdenii*, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Joyner et al., 1977; Van Dijk et al., 1979). The presence of hydrogenases has also been reported in ruminal protozoa and fungi (Paul et al., 1990; Yarlett et al., 1981; Yarlett et al., 1986).

To our knowledge, hydrogenases composition has poorly been studied. Using radioactivity, it was reported that *Wolinella succinogenes* owns a [Ni-Fe] hydrogenase (Unden et al., 1982). Recent sequencing of cDNA coding for a small piece of hydrogenase ("H-cluster") showed that M*egasphaera elsdenii*, several species of the genus *Desulfovibrio* and rumen eukaryotes host [Fe-Fe] hydrogenases. Phylogenetic analyses also revealed that

there is few relationship between [Fe-Fe] hydrogenases from prokaryotes and eukaryotes (Boxma et al., 2007).

### *2.3. Balance of hydrogen production in the rumen during microbial feed fermentation*

Within rumen microbes, hydrogenases are responsible for the oxidation of two moles reduced Fed while producing one mole $H_2$ (Gottschalk, 1986):

$$2\ Fed_{red} + 2H^+ + 2e^- \leftrightarrow 2Fed_{ox} + H_2$$

Consequently, we can now calculate the molar production of $H_2$ during carbohydrates fermentation (Table 3). The production of one mole acetate or one mole butyrate from one mole glucose generates 2 moles $H_2$ whereas 1 mole $H_2$ is required to produce one mole propionate. These results are similar to Sauvant et al., 2011. As eukaryotes preferentially ferments glucose to acetate and butyrate (Williams and Coleman, 1997), they are considered as important $H_2$-producers.

**Table 3** Molar $H_2$ production during fermentation of one mole glucose

| VFA | Moles from one mole glucose | Reduced ferredoxin production (moles) | $H_2$ production (moles) |
|---|---|---|---|
| Acetate | 2 | +8 | +4 |
| Butyrate | 1 | +4 | +2 |
| Propionate | 2 | -4 | -2 |

Concerning protein fermentation, the balance of $H_2$ production is less evident to calculate as it is dependent on AA profiles. However, it has been estimated that when microbes grow on AA as the sole N source, $H_2$ would be produced at a rate of 0.58 moles per kilogram of microbes, assuming a microbial composition of 53 g protein/100g dry microbial matter (Mills et al., 2001).

## III. RUMEN HYDROGEN SOLUBILITY AND CONCENTRATION

After its production, $H_2$ diffuses through the cell cytoplasmic membrane to the ruminal environment in a dissolved form. The diffusion rate is dependent on the microbial cell physiology (cell size and form) and on the external $H_2$ concentration: the higher the external dissolved $H_2$ concentration, the lower is the diffusion rate of $H_2$ out of the cell. This maintains an equilibrated gradient between the cell and its environment (Boone et al., 1989). The

external $H_2$ concentration is in turn an equilibrium between dissolved and gaseous $H_2$ concentrations.

## *3.1. Dissolved hydrogen concentration in the rumen liquid phase*

### 3.1.1. Hydrogen solubility and maximum theoretical concentration

The theoretical maximum $H_2$ concentration in the liquid phase of the rumen (dissolved $H_2$) is related to its solubility. Hydrogen solubility in water (µ*M*/atm) is a function of temperature (T, K) and salinity (S, ‰). Its calculation involves the determination of Bunsen solubility coefficient (β, ml dissolved $H_2$ in 1 mL $H_2O$; Wiesenburg and Guinasso, 1979):

$$\ln \beta = \mathrm{A1} + \mathrm{A2} \times \frac{100}{T} + A3 \times \ln\left(\frac{T}{100}\right) + S \times (B1 + B2 \times \frac{T}{100} + B3 \times \frac{T}{100^2})$$

Where A1 = -47.8948, A2 = 65.0368, A3 = 20.1709, B1 = -0.082225, B2 = 0.049564 and B3 = -0.0078689.

Then, at ruminal temperature (39°C or 312K) and assuming a null salinity in the rumen, β is equal to 0.0166 ml $H_2$/ml $H_2O$. By applying the equation of ideal gas law in normal pressure ($1.01325 \times 10^5$ Pa) and temperature (273K) conditions, the β solubility of $H_2$ is 740.9 µ*M*. Consequently, the maximum concentration of dissolved $H_2$ in the rumen is 740.9 µ*M* assuming there is no other dissolved gas in the liquid. This result is coherent, knowing the standard $H_2$ solubility (759 µ*M*) at 30°C in water with zero salinity (Wiesenburg and Guinasso, 1979).

### 3.1.2. Observed rumen dissolved hydrogen concentration

*Measure of dissolved $H_2$ concentrations.* Owing to the high volatility of $H_2$ and its high turnover time (0.08 sec; Smolenski and Robinson, 1988), the dosage of dissolved $H_2$ concentrations is not easy. In the literature, two studies succeeded to measure *in situ* dissolved $H_2$ concentrations in the rumen. In the first one (Hillman et al., 1985), dissolved $H_2$ diffused in a Clark-type oxygen electrode placed within the rumen. Hydrogen concentration was determined *via* a mass spectrometer. In the second one (Smolenski and Robinson, 1988), dissolved $H_2$ was uptaken by a carrier gas (helium) passing through a probe immerged into the rumen. The gas mixture was then heated in order to separate helium from $H_2$, and $H_2$ concentration was measured with a gas chromatograph.

Other developed methods are based on point-by-point analysis by gas chromatography of gas extracted from rumen juice. Rumen fluid was sampled in a syringe and $H_2$ was extracted *via* two methods: i) $H_2$ was gasified by heating the sample (Hungate, 1967) or by injecting the sample into a basic solution (Robinson et al., 1981); ii) Nitrogen (N) was diffused into the sample and after mixing and collection of upper gas, $H_2$ concentration was determined according to N dilution (Czerkawski and Breckenridge, 1971; Wang et al., 2014).

*Observed ruminal dissolved $H_2$ concentrations.* In a normal functioning rumen and outside feeding time, the basal concentration of dissolved $H_2$ is low, ranging between 0.6 and 3.4 µ*M* (Table 4). This corresponds to a range between 0.081 and 0.459% of its maximal solubility. Two factors induce variations in these concentrations: the diet composition and the feeding time (Janssen, 2010). Dissolved $H_2$ concentrations increased from 2 to 3 hours postfeeding due to the increase in fermentation (Figure 9; Czerkawski and Breckenridge, 1971). This postfeeding rise is all the more important as diets are rich in quickly and readily fermentable feed (e.g. high grain diets).

**Table 4** Dissolved hydrogen concentration in the rumen of ovine or cattle.

| Reference | Animal species | Diet | Dissolved $H_2$ concentrations |
|---|---|---|---|
| Hungate (1967) | Bovine | 100% lucerne hay | 0.6-1.3 µ*M* |
| Hillman et al. (1985) | Ovine | 100% grass hay | 0.6-3.4 µ*M* |
| Smolenski et Robinson (1988) | Bovine | High forage diet (composition not mentioned) | 1-1.4 µ*M* (20 µ*M* 10 min postfeeding) |
| Robinson et al. (1981) | Bovine | 75% grain + 25% hay | 1 µ*M* (15 µ*M* 1 h postfeeding) |
| Czerkawski et al. (1971) | Ovine | Molassed sugar beet before $H_2$ measurement (complete diet not mentioned) | 48 µ*M* (20 min postfeeding<br>1-2 µ*M* (5 h postfeeding) |
| Morgavi et al. (2012) | Ovine | 58% lucerne pellet + 25% cracked maize grain + 17% prairie hay | 22.6 µ*M* (3 h postfeeding) |

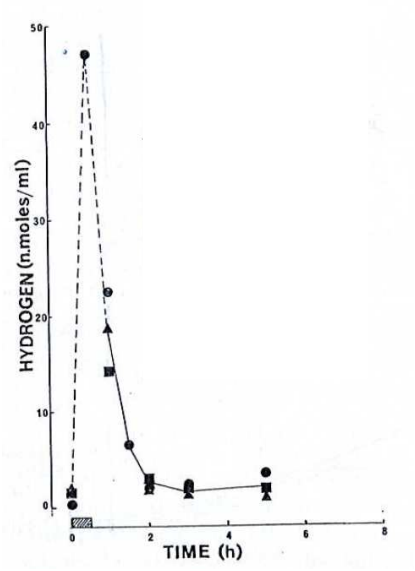


**Figure 9** Ruminal dissolved hydrogen concentrations of sheep given 500 g molassed sugar beet pulp at time 0 (from Czerkawski and Breckenridge, 1971)

### *3.2. Equilibrium between dissolved and gaseous hydrogen in the dorsal sac*

#### 3.2.1. Theoretical equilibrium between dissolved and gaseous hydrogen

The presence of a dissolved gas in a liquid phase necessarily involves the presence of its gaseous form. Then, according to the dissolved $H_2$ concentration in the rumen, it may be possible to calculate the theoretical partial pressure of gaseous $H_2$ in the dorsal sac of this digestive compartment according to the Henry's law (Sander, 1999):

$$K_H = \frac{c_a}{p_g}$$

Where $K_H$ is the Henry's law constant (*M*/atm), $c_a$ is the concentration of $H_2$ in the liquid phase (*M*) and $p_g$ is the partial pressure of $H_2$ in the gaseous phase (atm).

The Henry's law constant $K_H$ depends on the medium temperature as the equilibrium between dissolved and gaseous phase is dependent on this parameter:

$$K_H = K_H^\theta \times \exp\left[-A\left(\frac{1}{T} - \frac{1}{T^\theta}\right)\right]$$

Where $K_H^\theta$ is $K_H$ at standard temperature conditions ($K_H^\theta = 7.8\times10^{-4}$ M/atm), A is a constant depending on the enthalpy of the solution (A = 500K), T is the temperature in the medium and $T^\theta$ is the standard temperature ($T^\theta$ = 298K). Finally, at rumen temperature (T = 312K), $K_H$ is equal to 0.000841 *M*/atm. Consequently, if dissolved $H_2$ concentrations reach its maximum ($c_a$ = 740.9 µ*M*), the theoretical partial pressure of $H_2$ would be 0.88 atm (88% $H_2$).

However, a recent *in vitro* ruminal study showed that an increase of dissolved $H_2$ concentrations is not necessarily linked with an increase of gaseous $H_2$ (Wang et al., 2014). In that study, the authors concluded that the equilibrium between dissolved and gaseous $H_2$ may not completely respect Henry's law, probably because of mass-transfer[3] limitation. Indeed, the transfer of $H_2$ from the rumen liquid phase to the rumen gaseous phase may be affected by the diffusity coefficient of this gas and by the mixing efficiency of this digestive compartment (Pauss et al., 1990). Then, $H_2$ may accumulate in certain part of the rumen, limiting the possibility to calculate gaseous $H_2$ concentrations from dissolved $H_2$ concentrations measured in one part of the rumen, and *vice versa*. This also highlights the importance of *in vivo* measurement of $H_2$ concentrations in both phases. Nonetheless, to our knowledge, such experiment has still not been carried out.

### 3.2.2. Observed hydrogen concentrations in the rumen gaseous phase

*Measure of gaseous $H_2$ concentration in the dorsal sac of the rumen.* Several methods have been applied to measure $H_2$ concentrations in the rumen gaseous phase. With non-canulated cows, gas has always been sampled by rumenocentesis, and gas composition was analyzed by gas chromatography (Jouany and Senaud, 1979; McArthur and Miltimore, 1961; Moate et al., 1997; Moate et al., 2013; Moate et al., 2014) or by the Orsat gas analyzer[4] (Olson, 1940). With cannulated cows, gas has been collected with a bag attached to the cannula and filled thanks to rumen contraction (Barry et al., 1977) or with a syringe inserted through the plug of the rumen cannula (Moate et al., 2013). Gas composition was analyzed by gas chromatography.

*Observed gaseous $H_2$ concentrations.* Partial pressure of $H_2$ in the gaseous phase of bovine and ovine rumen ranges between 0.023 and 26.5% (Table 5). Several factors may explain the within-experiment variability. Gaseous $H_2$ concentrations are higher during the 2 h following meals (Barry et al., 1977; Jouany and Senaud, 1979) and when rapidly-degradable substrates are fed (Barry et al., 1977). Bloated animals after legumes feeding may have higher gaseous $H_2$ proportions, probably linked with a rumen dysfunction (Olson, 1940). However, Moate et al. (1997) did not observe differences in gaseous $H_2$ between bloated and non-

[3] Mass transfer is defined as the movement of a mass from one phase to another.
[4] The Orsat gas analyser system is based on absorption of gases of interest by specific chemical solutions.

bloated dairy cows. Between-experiments variability in gaseous $H_2$ concentrations may be explained by the presence or absence of rumen cannula: cannulated animals have lower $H_2$ proportions than non-cannulated animals, probably because of air exchange *via* the cannula between the rumen and its external environment (Moate et al., 2013).

**Table 5** Composition of rumen headspace gas (adapted from Hegarty and Gerdes, 1999)

| Reference | Animal species | Diet | Rumen headspace gas composition (%) | | |
|---|---|---|---|---|---|
| | | | $CO_2$ | $CH_4$ | $H_2$ |
| Olson, 1940 | Bovine | Sweet clover | | | |
| | | - Non bloated | 60.7 | 0.14 | 9.36 |
| | | - Bloated | 62.0 | 15.3 | 0.31 |
| | | Alfalfa | | | |
| | | - Non bloated | 53.5 | 0.05 | 26.5 |
| | | - Bloated | 59.8 | 18.4 | 0.05 |
| McArthur and Miltimore, 1961 | Bovine | Unspecified | 65.4 | 26.8 | 0.18 |
| Barry et al., 1977 | Ovine | 100% hay | | | |
| | | - before feeding | 47.1 | 36.2 | 0.033 |
| | | - feeding time | 24.5 | 12.0 | 0.046 |
| | | - 2 h postfeeding | 47.5 | 33.0 | 0.062 |
| | | 80% hay, 20% concentrate | | | |
| | | - before feeding | 54.3 | 26.1 | 0.023 |
| | | - feeding time | 35.7 | 16.3 | 0.319 |
| | | - 2 h postfeeding | 68.4 | 24.8 | 0.135 |
| Jouany and Senaud, 1979 | Ovine | 40% dehydrated lucerne, 9% wheat straw, 51% concentrate | | | |
| | | - 1 h postfeeding | 62.6 | 32.0 | 1.68 |
| | | - 5 h postfeeding | 57.3 | 32.8 | 1.10 |
| | | - 10 h postfeeding | 45.0 | 35.7 | 0.05 |
| Moate et al., 1997 | Bovine | White clover pasture | | | |
| | | - Non bloated | 75.8 | 23.1 | <0.10 |
| | | - Bloated | 75.0 | 23.5 | <0.10 |
| Moate et al., 2013 | Bovine | 54% grain, 46% alfalfa hay | | | |
| | | - Non-cannulated | 49.8 | 26.1 | 0.14 |
| | | - Cannulated | 13.4 | 3.8 | 0.03 |
| Moate et al., 2014 | Bovine | Alfafa hay (AH), grain, dry or ensiled grape marc (DGM or EGM) | | | |
| | | - 76% AH, 24% grain | 62.8 | 37.1 | 0.11 |
| | | - 50% AH, 27% DGM, 23% grain | 60.1 | 39.8 | 0.13 |
| | | - 50% AH, 27% EGM, 23% grain | 61.3 | 38.6 | 0.11 |
| **Average** | | | **54.4** | **23.9** | **2.1** |
| **SD** | | | **15.31** | **12.29** | **6.27** |

SD: Standard deviation

# CHAPTER 2: Methanogenesis, not a unique pathway using hydrogen in the rumen

Hydrogenases activity can be inhibited by an accumulation of $H_2$ in their environment, with bacterial hydrogenases ([Ni-Fe] hydrogenases) being even more sensitive than protozoal hydrogenases ([Fe-Fe] hydrogenases) (Fourmond et al., 2013). Consequently, to ensure continuity of fermentation in the rumen, it is essential to maintain a low $H_2$ concentration *via* efficient mechanisms of removal and uptake of $H_2$.

## I. METHANE PRODUCTION

Methane production is the main pathway using $H_2$. Czerkawski (1986) estimated that 48% of produced $H_2$ would be used towards this pathway. With a different approach, a more recent mechanistic model even increased this percentage to 80% with the assumption that methanogenesis uses the excess of $H_2$ which has not been used by other $H_2$ using pathways (Mills et al., 2001).

In the rumen, hydrogenotrophic methanogens use $H_2$ as an energy source for their growth while producing $CH_4$:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

The linear and positive relationship between rumen $H_2$ concentration and $CH_4$ production has been emphasized in four *in vitro* experiments (Czerkawski et al., 1972; Hungate, 1967; Wang et al., 2014; Zaiß and Kaltwasser, 1979): correlation between dissolved $H_2$ and $CH_4$ concentration in headspace would average 0.92 (Wang et al., 2014) and Zaiß and Kaltwasser (1979) reported a correlation of 0.90 between hydrogenase activity and methanogenesis.

The microbial mechanisms under $CH_4$ production involve interspecies $H_2$ transfer between $H_2$-producers and methanogens (Wolin et al., 1997). The most studied example of this $H_2$ transfer is the symbiotic relationship between methanogens and protozoa (Finlay et al., 1994; Newbold et al., 1995; Stumm et al., 1982; Ushida and Jouany, 1996; Vogels et al., 1980): methanogens are positioned on the protozoa to reduce the distance for diffusion of $H_2$ from the hydrogenosome. These methanogens associated with protozoa would be responsible for between 9 and 25% of methanogenesis in rumen fluid (Newbold et al., 1995). A recent analysis of the literature highlighted a positive relationship between protozoa and $CH_4$

emissions: a reduction of 0.12 $log_{10}$ protozoa cells/mL would reduce $CH_4$ by 1 g/kg DMI (Morgavi et al., 2010). By an *in vitro* approach, *Entodinium* species were found to be the protozoal genus contributing the most to $CH_4$ emissions, followed by *Epidinium caudatum*. *Polyplastron* had the lowest contribution (Newbold et al., 1995).

## II. VOLATILE FATTY ACIDS SYNTHESIS

Volatile fatty acids synthesis would be responsible for 19-33% of the $H_2$ uptake (Czerkawski, 1986; Mills et al., 2001). Only propionate and valerate formation uses $H_2$, with one mole $H_2$ required per mole produced propionate or valerate.

Two propionate precursors have been tested to reduce $CH_4$ emissions. Firstly, based on stoichiometry, the conversion of one mole fumarate to propionate would reduce $CH_4$ emissions by 5.6L (Newbold et al., 2005). However, fumaric acid tested *in vivo*, showed a low and variable anti-methanogenic effect which is not dose-dependent (4% $CH_4$ reduction per percent added fumaric acid, on average). A reduction of $CH_4$ emissions (g/kg DMI) of 21.8% was reported when supplying 2.0% of fumaric acid to male steers (Bayaru et al., 2001). In beef cattle, $CH_4$ emissions (g/kg DMI) raised by 10.2% while feeding 2.4% of fumaric acid (Beauchemin and McGinn, 2006). In dairy cattle, 2.5% of fumaric acid did not affect $CH_4$ emissions (g/kg DMI; Van Zijderveld et al., 2011a). In sheep supplied with 10% of fumaric acid, $CH_4$ emissions (g/kg DMI) were reduced by 57% (Wood et al., 2009). The contradictory $CH_4$ mitigating effect of fumarate was also reported when analyzing several *in vitro* experiments by a meta-analysis approach (Ungerfeld et al., 2007). These authors calculated that only 48% of added fumarate would be converted into propionate, confirming previous results (Newbold et al., 2005). They assumed that this incomplete conversion of fumarate may be caused by its rapid disappearance in the rumen.

Secondly, the anti-methanogenic effect of malic acid seems to be low (2% $CH_4$ reduction per percent malic acid, on average), but more repeatable with doses equal or higher than 2%. Malic acid at a dose of 1.2% did not affect $CH_4$ emissions (g/kg DMI) of dairy cows (Doreau et al., 2014b). With a dose of 2.0% fed to male steers, malic acid reduced $CH_4$ emissions (g/kg DMI) by 17.3% (Lila et al., 2004). Using malic acid at doses of 3.5 and 7.5%, $CH_4$ emissions (g/kg DMI) of heifers were linearly reduced from 2.7% to 9.2%, respectively (Foley et al., 2009).

## III. MICROBIAL BIOMASS SYNTHESIS

According to calculations, bacteria and protozoa would be composed of 6.23 H atoms/100 g cells (Reichl and Baldwin, 1975; Table 6). Then, $H_2$ is essential for microbial synthesis, but their requirement level is variable in the literature. Czerkawski (1986) estimated that 12% of produced $H_2$ is used for microbial growth. In the model of Mills et al. (2001), this percentage is much lower, considering that 0.6% of $H_2$ would be directed towards microbial growth. This important difference between the two studies must come from the different ways of calculation of microbial composition. Mills et al. (2001) estimated that microbes require $H_2$ only when they grow with non-protein nitrogen (NPN), and this requirement was assessed at 0.41 moles $H_2$ per kilogram of microbes. This requirement level has been set considering polysaccharide-free microbial dry matter, whereas previous studies took into account the storage polysaccharide (Benchaar et al., 1998). Consequently, in order to precisely assess the amount of $H_2$ used for microbial biomass synthesis, it will be necessary to standardize the methods of calculation.

The between-experiment variability in $H_2$ requirement for microbial growth may also come from the level of nutrients deficiency in the diets. Indeed, when the crude protein (CP) content of the diet is low, microbes have to use NPN source, which increases microbial growth efficiency and then, $H_2$ uptake (Leng, 2014).

**Table 6** Bacterial composition (from Reichl and Baldwin, 1975)

| | Protein | Nucleic acid | Polysaccharide | Lipid | Ash |
|---|---|---|---|---|---|
| Bacteria (g/100g dry cells) | 54.46 | 9.08 | 20.16 | 11.54 | 4.76 |
| Bacteria (mol/100g cells) | 0.474 | 0.028 | 0.124 | 0.019 | -- |
| Hydrogen (atoms/mol) | 7.59 | 12 | 10 | 55.8 | -- |

## IV. BIOHYDROGENATION OF POLY-UNSATURATED FATTY ACIDS

Czerkawski (1986) and Mills et al. (2001) estimated that only between 1 and 2.6% of $H_2$ is uptaken for biohydrogenation, which consists in H saturation of double bonds of unsaturated fatty acids. This means that the reduction in $CH_4$ emissions observed in several experiments testing polyunsaturated fatty acids (PUFA) in ruminants diets (Beauchemin and McGinn, 2006; Beauchemin et al., 2009; Chung et al., 2011; Martin et al., 2008) cannot be solely explained by biohydrogenation.

For instance, we can assume that a complete saturation of oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) requires 1, 2 and 3 moles $H_2$, respectively. When applying these coefficients to the experiment of Martin et al. (2008), feeding 5.8% linseed oil (49.2% C18:3; 21.3% C18:2; 15.1% C18:1) to lactating cows eating 14.7 kg DM would reduce $CH_4$ emissions by 25.1 g/day. However in this experiment, $CH_4$ was reduced by 268.9 g/day which was 10 times more than theoretically calculated, showing the absence of relationship between the quantity of saturated double bonds and the extent of $CH_4$ inhibition. In other words, this difference highlights that the $CH_4$ mitigating effect of PUFA is only partly due to $H_2$ uptake for biohydrogenation. Other reasons may explain the negative effects of lipids on methanogenesis. PUFA must have a toxic effect on protozoa which are important $H_2$ producers (Doreau and Ferlay, 1995). The degradation of diet digestibility with high doses of PUFA (more than 5% added fat in Martin et al., 2008) must reduce $H_2$ production and availability for methanogens. As lipids are mostly digested in the intestine, $H_2$ production in the rumen is reduced when fed in substitution of carbohydrates.

## V. OTHER HYDROGEN-SINKS COMPETING METHANOGENESIS

In aerobic environment, oxygen ($O_2$) is the most important $H_2$-sink, due to its high affinity for $H_2$ ($O_2 + 2H_2 = 2H_2O$). Inversely, in anaerobic environment, $CO_2$, propionate precursors (Hattori and Matsui, 2008; Henderson, 1980; Reddy and Peck, 1978), nitrate, sulfate (Laverman et al., 2012; Van Zijderveld et al., 2010), iron or manganese (Lovley, 1991; Nealson and Saffarini, 1994) can play the role of $H_2$-sink. When all these electrons acceptors are present in an anaerobic environment, thermodynamic laws define the ranking of molecules reduction.

### *5.1. Thermodynamic laws governing the affinity of electrons acceptors for hydrogen*

Reactions between $H_2$ and electrons acceptors are oxidation-reduction reactions, which involve two redox couples exchanging electrons. Each couple is composed of an oxidant (Ox) and a reducer (Red):

Couple 1 ($Ox_1/Red_1$): $Red_1 = Ox_1 + ne^-$ (Oxidation)

Couple 2 ($Ox_2/Red_2$): $Ox_2 + ne^- = Red_2$ (Reduction)

Final equation balance: $Red_1 + Ox_2 = Ox_1 + Red_2$

Each redox couple is characterized by an equilibrium constant between the oxidant and the reducer, named the "standard reduction potential" ($E_0$, V) which measures the tendency of the reducing agent to lose electrons (

Table **7**). The exchange of electrons between two couples is spontaneously possible if the variation $\Delta E_0$ between their standard reduction potential is positive (exergonic reaction):

$$\Delta E_0 = E_0 \text{ (Reduction)} - E_0 \text{ (Oxidation)} > 0$$

Should this not be the case ($\Delta E_0 < 0$), the reaction would require energy (endergonic reaction). The affinity between two redox couples is determined by the "Gibbs free energy" ($\Delta G$) liberated during their reaction:

$$\Delta G = -nF \times \Delta E_0$$

Where n = number of electrons involved in the process, F = Faraday constant (96.500 kJ/V/mol) and $\Delta E_0$ = the difference of standard reduction potentials between the two redox couples (V). In spontaneous process, $\Delta G$ is negative and the lower it is, the higher will be the free energy liberated. This means that redox couples with negative $E_0$ will tend to give electrons to redox couples with the more positive $E_0$. Then, in an given environment, $H_2$ will have a decreasing affinity for $O_2$, $NO_3^-$, $MnO_4^-$, $Fe^{3+}$, Fumarate, $SO_4^{2-}$ and $CO_2$ (Table 8).

**Table 7** Standard reduction potentials of several common redox couples at pH = 7 (Prescott et al., 2010; Tratnyek and Macalady, 2000)

| Redox couples | Reduction half-reaction | $E_0$ (V) |
|---|---|---|
| $H^+/H_2$ | $2H^+ + 2e^- \rightarrow H_2$ | -0.42 |
| $Fed_{ox}/Fed_{red}$ | $Fed_{ox} + e^- \rightarrow Fed_{red}$ | -0.42 |
| $NAD(P)^+/NAD(P)H$ | $NAD(P)^+ + 2H^+ + 2e^- \rightarrow NAD(P)H + H^+$ | -0.32 |
| $CO_2/CH_4$ | $CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$ | -0.25 |
| $SO_4^{2-}/HS^-$ | $SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O$ | -0.21 |
| $FAD/FADH_2$ | $FAD + 2H^+ + 2e^- \rightarrow FADH_2$ | -0.18 |
| Fumarate/Succinate | $HOOCCH{=}CHCOOH + 2H^+ + 2e^- \rightarrow HOOC(CH_2)_2COOH$ | +0.03 |
| $NO_3^-/NO_2^-$ | $NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$ | +0.42 |
| $Fe^{3+}/Fe^{2+}$ | $Fe^{3+} + e^- \rightarrow Fe^{2+}$ | +0.77 |
| $O_2/H_2O$ | $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$ | +0.82 |
| $MnO_4^-/Mn^{2+}$ | $MnO_4^- + 8H^+ + 5e^- \rightarrow Mn^{2+} + 4H_2O$ | +0.84 |
| $NO_2^-/NH_4^+$ | $NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$ | +0.90 |

**Table 8** Gibbs free energy liberated between $H_2$ and several electron acceptors

| Redox couples | ΔG (kJ) |
|---|---|
| $O_2/H_2O$ | -239 |
| $NO_3^-/NO_2^-$ | -162 |
| $MnO_4^-/Mn^{2+}$ | -122 |
| $Fe^{3+}/Fe^{2+}$ | -115 |
| Fumarate/succinate | -87 |
| $SO_4^{2-}/HS^-$ | -41 |
| $CO_2/CH_4$ | -33 |

### *5.2. Electrons acceptors tested in rumen*

The rumen being an anaerobic environment, $O_2$ cannot be used to oxidize $H_2$. Others electrons acceptors have been tested in the rumen to reduce methanogenesis with the hypothesis that they can efficiently compete for $H_2$, reducing its availability for $CH_4$ production. To our knowledge, fumarate (mentioned above), nitrate and sulfate are the only other electrons acceptors which have been tested *in vivo*.

Four moles $H_2$ would be used in the reduction of 1 mole nitrate to 1 mole ammonia (*via* nitrite production by a periplasmic reductase; Iwamoto et al., 2001; Simon et al., 2003) or in the reduction of 1 mole sulfate to 1 mole hydrogen sulfide. Consequently, knowing that 4 moles $H_2$ are also required to produce 1 mole $CH_4$, theoretically, 1 mole nitrate or sulfate in diets would reduce $CH_4$ production by 1 mole (22.4 L). In *in vivo* experiments, nitrate or sulfate effectively reduced $CH_4$ production (Table 9). Methane reduction efficiency, calculated as the ratio between observed $CH_4$ emissions and expected $CH_4$ emissions based on stoichiometry, ranged between 42 and 119%. The inefficient use of nitrate and sulfate may be explained by the higher proportion of acetate in the rumen of animals supplemented with nitrate, which synthesis produces $H_2$ counteracting the reduction of $H_2$ availability caused by these two chemicals (Nolan et al., 2010; Hulshof et al., 2012; Veneman et al., 2014).

**Table 9** Efficiency of methane reduction when supplementing ruminants with electrons acceptors.

| Reference | Animal species | Electron acceptor | Dose (% DM) | $CH_4$ reduction (g/kg DMI) | | Efficiency[1] (%) |
|---|---|---|---|---|---|---|
| | | | | Expected | Observed | |
| Van Zijderveld et al., 2010 | Ovine | Nitrate | 2.6 | 6.7 | 5.9 | 89 |
| Van Zijderveld et al., 2011 | Cattle | Nitrate | 2.1 | 5.4 | 3.0 | 56 |
| Hulshof et al., 2012 | Cattle | Nitrate | 2.2 | 5.7 | 6.1 | 107 |
| Nolan et al., 2012 | Ovine | Nitrate | 2.5 | 6.5 | 4.8 | 74 |
| Veneman et al., 2014 | Cattle | Nitrate | 2.0 | 5.2 | 4.6 | 89 |
| Veneman et al., 2014 | Cattle | Nitrate | 2.0 | 5.2 | 6.1 | 119 |
| Van Zijderveld et al., 2010 | Ovine | Sulfate | 2.6 | 6.7 | 2.8 | 42 |

[1] Efficiency was calculated as the ratio between observed *in vivo* $CH_4$ emissions and expected $CH_4$ emissions based on stoichiometry.

Supplementation of animals with nitrate or sulfate presents risks for their health, which explain why large scale use of these two chemicals is still not authorized in animal nutrition. Indeed, rapid ingestion by animals of high doses of nitrate may induce nitrite accumulation in the rumen which enters blood through the rumen wall, leading to the conversion of hemoglobin (Hb) to methemoglobin (metHb; Lewis, 1951). Contrary to Hb, metHb cannot transport oxygen and its accumulation may become life-threatening. Hydrogen sulfide coming from sulfate reduction may be eructated by the animal and re-enter the body during respiration. Inhalation of this gas by ruminants may induce polyoencephalomalacia which is a neurologic disorders characterized by necrosis of the cerebral cortex (Gould, 1998). Consequently, to counter the negative effects of nitrate and sulfate, it would be interesting to test novel electron acceptors.

Knowing the Gibbs free energy liberated during the redox reaction between $H_2$ and iron (Table 8), we assumed that iron III ($Fe^{3+}$) can also be an efficient electrons acceptor in the rumen, by diverting one electron from methanogenesis. We tested this hypothesis (unpublished data) using an automated *in vitro* rumen batch culture system (Muetzel et al., 2014). Five sources of iron (4 m*M*; iron II sulfate, iron II chloride, iron II acetate, iron III sulfate and iron III chloride) were incubated for 48 h with a substrate made of hay and concentrate (50:50) and a pasture-fed bovine inoculum. The iron II sources were used to know the outcome of iron II coming from iron III reduction. Both iron II and iron III sources reduced methanogenesis. Iron $CH_4$-mitigating efficiencies, calculated as the ratio between

expected $CH_4$ emissions based on stoichiometry and observed $CH_4$ emissions, ranged between 84 and 93% for iron II sources and averaged 84% for iron III sources.

As iron II presented the same $CH_4$-mitigating efficiency than iron III, we made two assumptions. Firstly, we assumed that iron may enhance another pathway using $H_2$ such as microbial biomass. To test this effect, iron II acetate (4 m*M*) and iron II chloride (4 m*M*) were incubated again for 48 h with glucose as the sole protein-free substrate to quantify the effect of iron on microbial growth. We observed that iron increased the concentration in insoluble proteins (Figure 12), indicating that iron may enhance $H_2$ uptake *via* a better microbial biomass synthesis. An additional dose-response study may highlight to which extent microbes are sensitive to iron availability. Anyway, knowing the low contribution of microbes in the use of $H_2$, other mechanisms must be involved in the $CH_4$-mitigating effect of iron.

Then, owing to the change in color of the medium within the first 10 h incubation (from green to dark black, Figure 10), we assumed that iron III and II are reduced in another form of iron while using electrons. In the rumen, knowing the average pH ([5.5;6.5]; Lettat, 2012) and Eh ([-150;-350]mV; Marden, 2007; personal database), diagrams of iron minerals indicate that iron should be in the form of vivianite ($Fe_3(PO_4)_2.8(H_2O)$) and/or magnetite ($Fe_3O_4$) (Figure 11). Then, to reduce iron III to iron, it may be 3 rather than 1 electron which would be deviated from methanogenesis.

**Figure 10** Color of the medium after 48 h incubation with hay and concentrate (50:50) supplemented with (left bottle) or without (right bottle) iron sources.

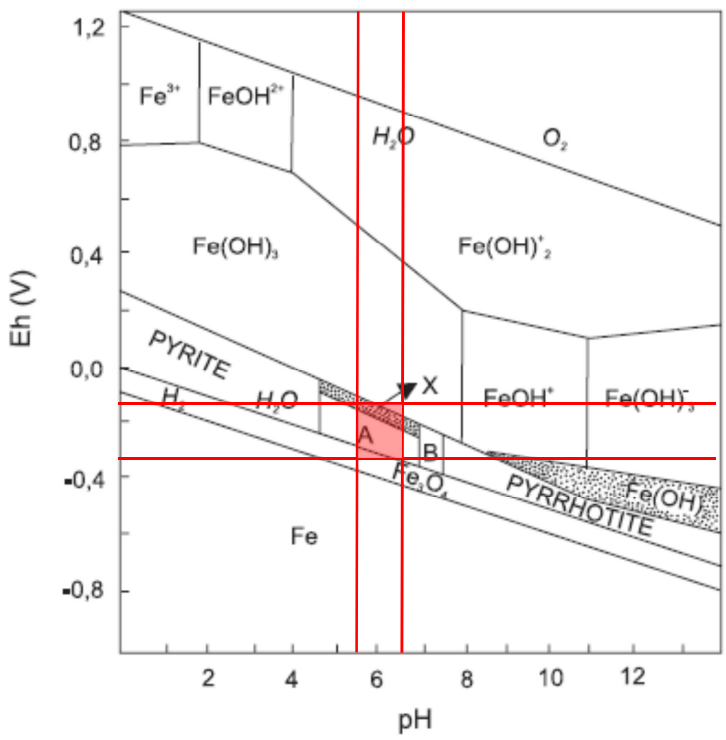


**Figure 11** Forms of iron minerals according to Eh and pH (A: vivianite; B: siderite; Lemos et al., 2007). Vertical and horizontal lines respectively correspond to pH and Eh ranges commonly found in ruminal conditions. The red square represents all the possible combinations of pH and Eh in the rumen, with associated forms of iron.

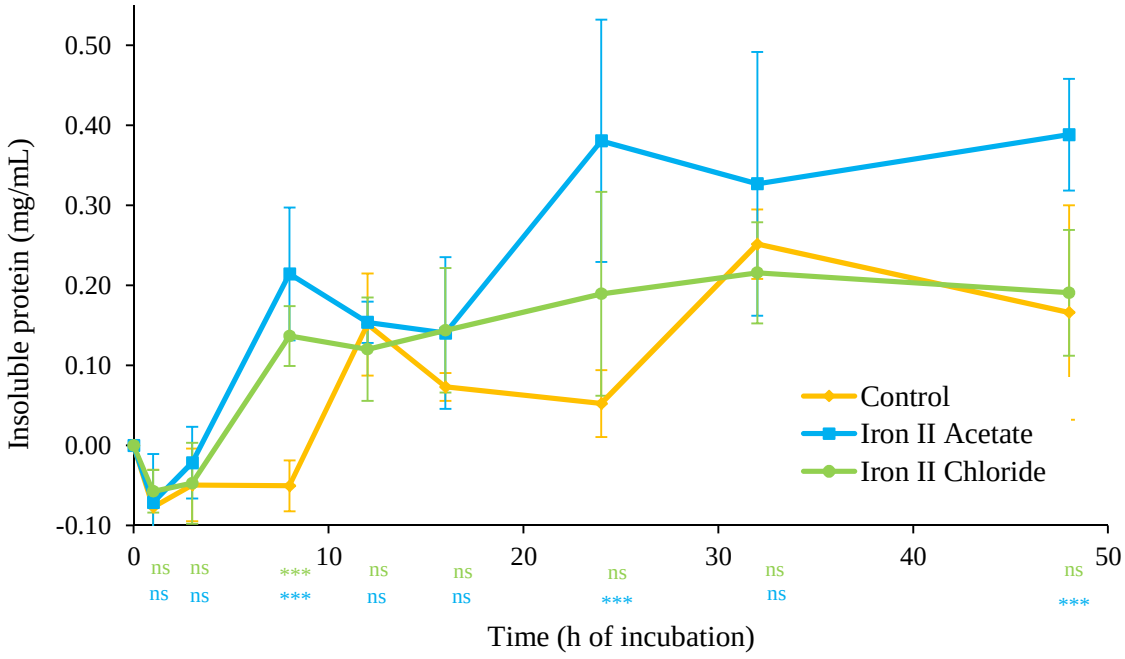


**Figure 12** Insoluble protein concentration during 48 h incubation with hay and concentrate (50:50) supplemented with iron II acetate (4 m*M*) and iron II chloride (4 m*M*). Error bars indicate standard deviation.

# CHAPTER 3: Emissions of gaseous hydrogen from the rumen: small energetic losses

Rumen stoichiometric models aiming to predict $CH_4$ emissions generally assume that the amount of $H_2$ produced is equal to the amount of $H_2$ used on a molar basis (Alemu et al., 2011; Benchaar et al., 1998; Mills et al., 2001). This hypothesis means that the $H_2$ recovery in $CH_4$, VFA and microbial synthesis would be equal to 100%, with no $H_2$ gas emitted from the animal. Consequently, few *in vivo* studies measured concentrations of $H_2$ emissions. However, results from these studies showed that $H_2$ emissions occur, even if they generally remain low, hardly detectable and represent a low percentage of GEI (less than 1% GEI).

## I. FACTORS OF VARIATION OF GASEOUS HYDROGEN EMISSIONS

### *1.1. Measurement of hydrogen emissions*

In the literature, two methods have been used to quantify gaseous $H_2$ emissions. In both of them, animals were placed in respiratory chambers but these methods differed in terms of gas sampling method. The first one consisted in manual sampling of gas with a syringe in the chamber air intake and exhaust ducts (Van Zijderveld et al., 2011). In the second method, gas was automatically sampled *via* a shunt from the air intake and exhaust duct going directly to a gas analyzer (Pinares-Patiño et al., 2012a).

Gas composition was then analyzed by gas chromatography. Two detectors have been used, having different detection levels: an electrochemical $H_2$ detector with a detection level of 5 to 10 ppm (Pinares-Patiño et al., 2012a) and a Quintron Breathtracker with a detection level of 1 to 2 ppm (Van Zijderveld et al., 2011).

### *1.2. Factors influencing hydrogen emissions*

#### 1.2.1. Intake level and meals frequency

The higher the amounts of DMI, the lower are $H_2$ and $CH_4$ emissions (% GEI). Indeed, the comparison of gaseous emissions of sheep fed increasing amounts of forage (DMI ranging from 0.40 kg forage/day to 1.60 kg/day) showed that $CH_4$ and $H_2$ emissions were linearly

reduced: from 8.39% GEI to 6.02% GEI for $CH_4$ and from 0.052% GEI to 0.034% GEI for $H_2$ (Hammond et al., 2013).

A low frequency of meals distribution induces higher postfeeding peaks of $H_2$ emissions associated to lower $CH_4$ emissions. One study compared $H_2$ emissions of two groups of sheep fed the same diet (60:40 mixture of lucerne hay and wheat grain) distributed either two or eight times per day (Swainson et al., 2011; Figure 13). Daily $CH_4$ emissions were lower for sheep fed twice daily (3.47 vs 6.35% GEI) whereas $H_2$ emissions were similar between the two groups (0.061% GEI). However, sheep fed twice daily presented high peaks of $H_2$ emissions till 40 ppm one hour postfeeding, directly followed by peaks of $CH_4$ emissions (up to 180 ppm). Gaseous emissions recovered lower and basal value within 3 h for $H_2$ (0 ppm) and 7 h for $CH_4$ (40 ppm). Inversely, sheep fed eight times a day presented more regular gaseous emissions within a day, which never reached values higher than 15 ppm for $H_2$ and which ranged between 80 and 160 ppm for $CH_4$.

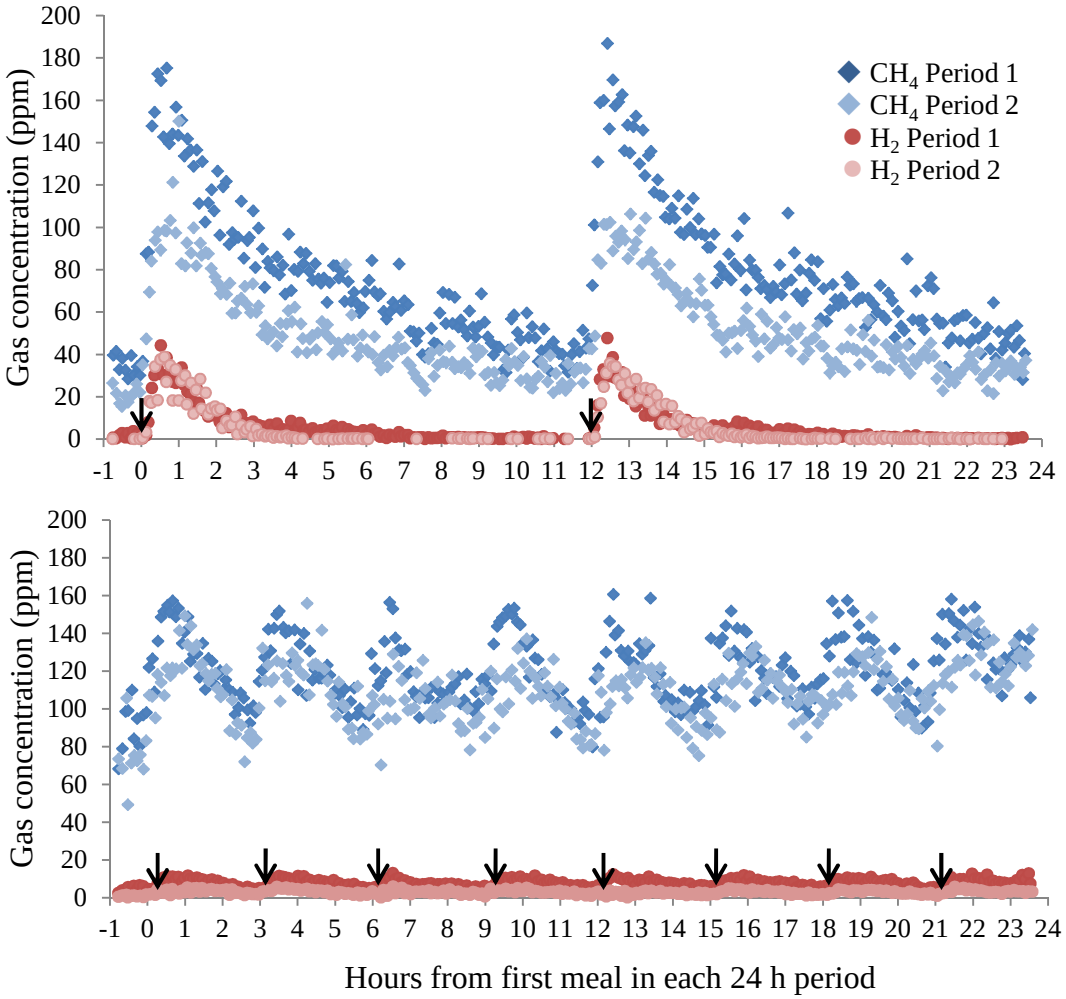


**Figure 13** Daily methane and hydrogen emissions kinetics (ppm) of sheep fed 2 (upper graph) or 8 (lower graph) times daily. The arrows indicate times of feeding. (from Swainson et al., 2011)

### 1.2.1. Diet composition and additives supplementation

High starch diets reduce $CH_4$ emissions without necessarily reducing $H_2$ emissions. A comparison between sheep fed either grass or a 60:40 mixture of lucerne hay and wheat grain showed that $H_2$ emissions from animals fed the high concentrate diet represented 0.115% GEI, which was six times more than the sheep fed grass (0.019% GEI) (Pinares-Patiño et al., 2010). Inversely, $CH_4$ emissions were lower for sheep fed the high concentrate diet (7.31 vs 11.66% GEI). However, it was recently shown that steers fed 92.5% of concentrates significantly emitted less $H_2$ than steers fed a mixed diet with 52% concentrates (1.47 vs 1.79% GEI) whereas $CH_4$ emissions (% GEI) were reduced by 37% with the high concentrate diet (Rooke et al., 2014).

Some chemicals supplemented in the diet of ruminants for reducing $CH_4$ emissions give rise to $H_2$ emissions. While inhibiting methanogenesis by 4.6% GEI with 0.2% of

hemiacetal of chloral and starch in diet, $H_2$ emissions were detected in rams up to 1.7% GEI (Johnson, 1972). Similarly, using the same inhibitor with half of previous dosage ($CH_4$ reduction of 3.94% GEI), $H_2$ emissions represented 0.8% GEI (Johnson, 1974). More recently, using 2.1% nitrate to reduce $CH_4$ by 1% GEI, dairy cows emitted more than 0.017% GEI, which was 2.5 times more than control cows (0.006% GEI; Figure 14; Van Zijderveld et al., 2011). When having a closer look to the kinetics, one may observe that the peak of gaseous $H_2$ caused by nitrate is situated 2 h postfeeding and 1 h before the postprandial peak of $CH_4$ emissions from control cows. Hydrogen release with nitrate supplementation may be explained by the punctual inhibiting effect of this chemical towards hydrogenotrophic methanogens (Iwamoto et al., 2001; Van Zijderveld et al., 2010).

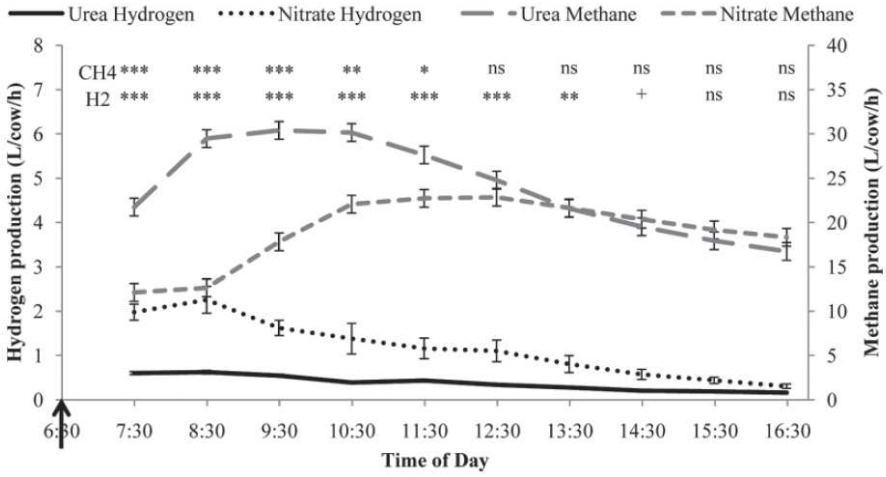


**Figure 14** Methane and hydrogen emissions kinetics of dairy cows supplemented with nitrate. The arrow indicates time of feeding. (from Van Zijderveld et al, 2011b)

Finally, $H_2$ emissions represent a low spoilage of energy (from 0.006 to 1.8% GEI) which is not used by the animal to produce VFA or microbial biomass. These low $H_2$ levels point out that the molecule is quickly metabolized in the rumen. The relationship between $H_2$ and $CH_4$ emissions is different between *in vitro* and *in vivo* experiments, as a positive and linear relationship has been reported *in vitro* between these two factors (chapter 2). *In vivo*, this relationship would be dependent on $H_2$ concentrations: above 0.1% GEI, a rise of $H_2$ emissions may be associated with a reduction of $CH_4$ emissions (Johnson, 1974, 1972; Pinares-Patiño et al., 2010; Van Zijderveld et al., 2011). Inversely, under 0.1% GEI, $H_2$ and $CH_4$ emissions are either not correlated (Swainson et al., 2011) or positively correlated (Hammond et al., 2013).

# II. CAUSES OF HYDROGEN EMISSIONS AND CONSEQUENCES

## *2.1. Two potential causes of hydrogen emissions*

To our knowledge, no *in vivo* experiment reported simultaneous measurements of dissolved $H_2$ in the liquid phase of the rumen, gaseous $H_2$ in the dorsal sac of the rumen and emissions of $H_2$ from the rumen. However, knowing the relationship between dissolved and gaseous $H_2$, we assume that an increase of $H_2$ emissions is linked to an evacuation of excessive gaseous $H_2$ coming from high dissolved $H_2$ concentrations in the liquid phase. Different scenarii may explain a build-up of $H_2$ in the rumen liquid phase: i) an increase in $H_2$ production with a constant $H_2$ use, ii/ a constant $H_2$ production with a lower $H_2$ use.

The first scenario may be applied in the case of an increase of DMI, a higher percentage of starch in diet or a lower feed frequency inducing the arrival of a large amount of feed in the rumen quickly fermented to $H_2$. In that case, the rate of production of $H_2$ may overload the capacity of methanogens to use $H_2$ (Rooke et al., 2014), therefore resulting in $H_2$ emissions (Swainson et al., 2011). Conversely, the second scenario may be applied while using anti-methanogenic strategies such as nitrate. Nitrate was shown to reduce the quantity of methanogens (Van Zijderveld et al., 2011), which are consequently not sufficient enough to compensate for the arrival of $H_2$ following ingestion.

## *2.2. Consequences of hydrogen emissions*

Hydrogen is an indirect GHG: it does not interact with solar and terrestrial radiations, but it perturbs the global distribution of important GHG such as $CH_4$ and ozone ($O_3$), by reacting with hydroxyl radicals. However, before considering $H_2$ emissions as a new source of pollution from ruminants, two factors have to be kept in mind. Firstly, despite some variations, $H_2$ emissions from ruminants remain at very low levels. Secondly, the GWP for $H_2$ is 5.8, which is much lower than $CH_4$ (GWP = 21) (Derwent et al., 2006).

# CHAPTER 4: Influence of rumen protozoa on methane emissions from ruminants: a meta-analysis approach

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**Abstract**

A meta-analysis was conducted to evaluate the effects of protozoa concentration on methane emission from ruminants. A database was built from 59 publications reporting data from 76 *in vivo* experiments. The experiments included in the database recorded methane production and rumen protozoa concentration measured on the same groups of animals. Quantitative data such as diet chemical composition, rumen fermentation and microbial parameters, and qualitative information such as methane mitigation strategies were also collected. In the database, 31% of the experiments reported a concomitant reduction of both protozoa concentration and methane emission (g/kg dry matter intake). Nearly all of these experiments tested lipids as methane mitigation strategies. By contrast, 21% of the experiments reported a variation in methane emission without changes in protozoa numbers indicating that methanogenesis is also regulated by other mechanisms not involving protozoa. Experiments that used chemical compounds as an antimethanogenic treatment belonged to this group. The relationship between methane emission and protozoa concentration was studied with a variance-covariance model, with experiment as a fixed effect. The experiments included in the analysis had a within-experiment variation of protozoa concentration higher than 5.3 $\log_{10}$ cells/ml corresponding to the average standard error of the mean of the database for this variable. To detect potential interfering factors for the relationship, the influence of several qualitative and quantitative secondary factors was tested. This meta-analysis showed a significant linear relationship between methane emission and protozoa concentration: $CH_4$ (g/kg dry matter intake) = -30.7 + 8.14 × protozoa ($\log_{10}$ cells/ml) with 28 experiments (91 treatments), root mean square error = 1.94 and adjusted $R^2$ = 0.90. The proportion of butyrate in the rumen positively influenced the least square means of this relationship.

**Keywords:** methane, protozoa, meta-analysis, ruminant, volatile fatty acids

**Implications**

Our meta-analysis allows the effect of a variation in rumen protozoa concentration on methane emission to be quantified when protozoa ranged between 4.5 and 7.3 $\log_{10}$ cells/ml. From selected experiments, a reduction of 0.12 $\log_{10}$ protozoa cells/ml induced a significant reduction of 1g methane/kg dry matter intake. Among the experiments of the database, 31% reported a reduction of both protozoa concentration and methane emission, most of these using lipids. However, a reduction of methane emission with no change in protozoa was

reported in 21% of the experiments, showing that protozoa are not the only factor responsible for reduced methanogenesis.

## Introduction

In the rumen, methanogens produce methane ($CH_4$) mainly from carbon dioxide ($CO_2$) and hydrogen ($H_2$) released during fermentation of feeds by bacteria, protozoa and fungi. Protozoa are involved in methanogenesis through their high production of butyrate (C4) and acetate (C2), two volatile fatty acids (VFA) whose biosynthesis liberates 2 and 4 moles of $H_2$ respectively, per mole of fermented glucose (Sauvant et al., 2011). Half of this $H_2$ is used by methanogens inside or in close association with protozoa cells, to produce $CH_4$ (Czerkawski, 1986; Williams and Coleman, 1992). Hence it was hypothesized that the reduction of rumen protozoa concentration might be an efficient way to decrease $CH_4$ emission (Finlay et al., 1994). Previous experiments testing experimental defaunation reported $CH_4$ reduction ranging from 13% to 35% *in vivo* (Hegarty, 1999; Morgavi et al., 2008; Morgavi et al., 2012) and from 9% to 25% *in vitro* (Newbold et al., 1995). However, the relationship between protozoa concentration and $CH_4$ emission is not precisely quantified. Preliminary work on a limited number of publications indicated that these two parameters were positively correlated (Morgavi et al., 2010). This finding prompted us to carry out a deeper analysis of the effects of a variation in protozoa concentration on $CH_4$ emission, by applying a meta-analysis approach with a variance-covariance model (Sauvant et al., 2008). To this end, we exhaustively gathered evidence from experiments reporting simultaneous measurements of $CH_4$ emission and rumen protozoa concentrations on the same groups of animals. To refine the study, we also tested the influence of qualitative and quantitative interfering factors for this relationship.

## Material and methods

### *Selection of publications*

We included in the database only publications reporting *in vivo* data of both $CH_4$ emission and rumen protozoa concentration measured on the same groups of animals. To find publications, bibliographical databases of editorial platforms (Web of Knowledge, ScienceDirect and Google Scholar) were interrogated, with methane, protozoa and ruminants as keywords. Unpublished experiments from our research group (INRA, UMR1213 Herbivores) were also added. Quantitative factors (intake, chemical composition of the diet, total tract digestibility,

rumen VFA concentrations, rumen pH, rumen bacteria and methanogen concentrations, and rumen sampling time relative to feeding time) were added to the database when available, with standard errors (s.e.) and statistical differences between treatments. Reported data of rumen parameters in kinetics were averaged. Qualitative factors (animal species, $CH_4$ mitigation strategies, and techniques for measuring $CH_4$ emission and protozoa concentration) were also collected. Publications using $CH_4$ emission calculated from equations instead of actual measures were excluded.

When relevant, treatments testing an additive or supplement were characterized by the main active compound in the additive (e.g. C18:1n-9 for rapeseed, diallyl disulfide for garlic, or tannin for *Quillaja saponaria*), by the quantity of the additive and of the main active compound in dry matter, and by the physical form of the additive (grain, powder, oil). For linseed, sunflower, rapeseed, soya, coconut and cottonseed, when the lipid values were not available, the quantity of the main fatty acid was calculated from tables of composition and nutritive value of raw ingredients (Sauvant et al., 2004). An experiment was defined as one control treatment and at least one experimental treatment testing one or several $CH_4$ mitigation strategies with the same basal diet. When relevant, one publication could supply different experiments, if controls were different. The final curated database contained 59 papers (number of experiments Nexp = 76, number of treatments Nt = 219) including 6 unpublished experiments (Nt = 24) from our research group. The list of published papers used is given in Supplementary material S1.

*Coding of experiments*

Experiments were first classified into four groups according to their $CH_4$ mitigation strategy. The group “biotechnology” (Nexp=13, Nt=35) consisted of experiments testing experimental defaunation, probiotics (*Lactobacillus*, *Propionibacterium*, *Saccharomyces*, *Trichosporon*), prebiotics (galacto-oligosaccharides) or exogenous microbial products (fibrolytic enzyme, secondary metabolites from *Monascus*). The group “additives” (Nexp=26, Nt=64) consisted of experiments testing chemical compounds (iodopropane, nitrate, sulfate), organic acids (malate, fumarate) or plants rich in tannin, saponin or essential oil (anacardic acid, diallyl disulfide, carvacrol, allyl isothiocyanate). The group “feed components” (Nexp=25, Nt=74) consisted of experiments testing lipids (C12:0, C14:0, C18:1n-9, C18:2n-6, C18:3n-3), forages (*Cichorium intybus*, *Lolium perenne*, *Trifolium repens*, *Trifolium pratense*, *Medicago sativa*, *Vigna unguiculata*) or cereal grains (wheat, maize, barley). The group “association” (Nexp=12, Nt=46) grouped experiments associating several strategies.

Experiments were further coded according to the distribution of the additive. Experiments with a "dose-response effect" tested different amounts of an additive (Nexp=41, Nt=105). Experiments with a "source effect" tested different sources of an active compound given at equal doses (e.g. the comparison between tannins originating from chestnut tree or acacia, Nexp=21, Nt=62). Experiments with a "form effect" tested different forms of an additive given at equal doses (e.g. the comparison between linseed fatty acids supplied as seed or oil, Nexp=2, Nt=6). Experiments testing experimental defaunation were considered as having a dose-response effect with protozoa as the active compound (Nexp=7, Nt= 17).

Experiments were then sorted into four classes according to their variations in $CH_4$ or protozoa: no variation of either parameters, variation in protozoa concentration only, variation in $CH_4$ emission only or variation in both parameters. Protozoa concentration was expressed as $\log_{10}$ cells/ml, to ensure normal distribution of residues. Three experiments reporting protozoa concentration as proportion of protozoal 18S rDNA per total bacterial 16S rDNA, or as log gene copies of protozoal 18S rRNA/g of fresh matter, could not be used, as conversion to $\log_{10}$ cells/ml was not possible. Methane emission were expressed in g per kg dry matter intake (DMI) to allow interpretation of data from animals with different levels of DM intake, i.e. large and small ruminants. Two papers had to be excluded, as DMI was not mentioned. Experiments were considered as reporting a significant variation in protozoa or $CH_4$ if the within-experiment variation of the parameter was respectively higher than one or two times the database average standard error of the mean (s.e.m.) for the parameter. The threshold levels for protozoa and $CH_4$ were then 5.3 $\log_{10}$ cells/ml ($2.2 \times 10^5$/ml) and 1.1 g/kg DMI, respectively.

*Statistical analysis*

*Description of the meta-design.* The relationship between the four $CH_4$ mitigation strategies and their effects on protozoa concentration or $CH_4$ emission was assessed with three chi square tests. The effect of the following classes on $CH_4$ mitigation strategies was tested: (i) variation in protozoa *vs.* no variation in protozoa, (ii) variation in $CH_4$ *vs.* no variation in $CH_4$, and (iii) variation in protozoa and/or $CH_4$.

In addition, the relationship between quantitative factors (see further) and rumen protozoa concentration ($\log_{10}$ cells/ml) or $CH_4$ emission (g/kg DMI) was examined to gain a better understanding of the effect of these factors on the relationship between $CH_4$ and protozoa. This analysis was performed using all the experiments in the database except for those testing defaunation, as they presented a high leverage effect. A one-way ANOVA was used to test

wether protozoa or $CH_4$ varied according to qualitative factors. In order to elucidate the relationship between protozoa or $CH_4$ and quantitative factors, various and complementary approaches were taken. Firstly, global correlation was calculated using all treatments irrespective of the experiment. Secondly, the between-experiment correlation was calculated using for each experiment, the mean of each factor and the mean of the protozoa concentration or of the $CH_4$ emission. Thirdly, the within-experiment correlation was calculated with a general linear model (GLM) using experiments with a reliable within-experiment variation of protozoa concentration or $CH_4$ emission:

$$Factor = \alpha + \alpha_i + \beta \times protozoa + \beta_i \times protozoa + e \quad [1]$$

$$Factor = \alpha + \alpha_i + \beta \times CH_4 + \beta_i \times CH_4 + e \quad [1']$$

where $\alpha$ = the overall intercept, $\alpha_i$ = the fixed effect of the experiment *i* on the overall intercept $\alpha$, $\beta$ = the overall slope, $\beta_i$ = the fixed effect of the experiment *i* on the slope and e = the random residual error.

*Response of $CH_4$ emission to a variation in rumen protozoa concentration.* The average response law was sought using experiments that had a sufficient variation in rumen protozoa concentration between control and treatment (average within-experiment variation of 5.3 $\log_{10}$ cells/ml). Five experiments using defaunated animals were excluded, as justified above. A GLM was applied to determine the relationship between $CH_4$ (g/kg DMI) and rumen protozoa concentration ($\log_{10}$ cells/ml):

$$CH_4 = \alpha + \alpha_i + \beta \times protozoa + \beta_i \times protozoa + e \quad [2]$$

where $\alpha$, $\alpha_i$, $\beta$, $\beta_i$, and e were as defined in equation 1. A quadratic adjustment was also tested and compared with the linear one. The experiment effect was included in the model as a fixed factor. Given that quantitative and qualitative factors differed between experiments and that they were not documented for all treatments, one of the major aims of this work was to study and explain how these factors might affect the relationship between protozoa and $CH_4$ emission. Normality of residuals was tested (Anderson-Darling test) and normalized residuals were calculated. Treatments with high normalized residuals (Nout, less than -3 or greater than +3) were identified and discarded from the model as statistical outliers if they also had a high leverage effect based on Hi calculation and Cook distance (Sauvant et al., 2008).

*Determination of factors influencing the response law.* Potential interfering factors for the response of $CH_4$ to protozoa were investigated. The interfering quantitative factors tested were: intake level (g DMI/day per kg BW), total tract digestibility of organic matter (OM),

NDF, starch and CP (%), rumen total concentration of VFAs (mmol/l), proportions of C2, C4 and propionate (C3) (mol/100mol), C2/C3 and (C2+C4)/C3 ratios (mol/mol), pH and concentrations of bacteria and methanogens (cells/ml) in rumen fluid. The interfering qualitative factors tested were: method of $CH_4$ measurement ($SF_6$, chamber), $CH_4$ mitigation strategy (biotechnology, additives, feed components, association), animal species (large or small ruminants), method of distribution of the additive (dose-response, source, form) and rumen sampling time (before feeding, i.e. more than six hours after last feeding; after feeding, i.e. less than six hours after last feeding; and average of before and after feeding).

The influence of these factors on the response law of $CH_4$ to protozoa was tested in a three-step process as described previously (Loncke et al., 2009). The first step consisted in highlighting the interfering factors influencing the three parameters of the model: slopes, least square means (LSMeans) and residuals (i.e. the difference between observed CH4 emission and emission predicted by the response law). A factor influencing the slopes or residuals may explain differences in variations of $CH_4$ emission between experiments for a similar variation in protozoa concentration. A factor influencing the LSMeans may explain the differences in $CH_4$ emission between experiments for a same level of protozoa. Slopes and LSMeans of each experiment used in the determination of the response law were calculated and their correlation with quantitative factors was tested. Residuals (observed minus predicted $CH_4$ emission) were calculated for all the treatments in the database, except for experiments testing defaunation, in order to ensure a normal distribution of the residuals. The relationship between residuals and quantitative factors was tested using the GLM procedure with experiment as a fixed factor:

$$Residuals = \alpha + \alpha_i + \beta \times factor + \beta_i \times factor + e \qquad [3]$$

where $\alpha$, $\alpha_i$, $\beta$, $\beta_i$ and e were as defined in equation 1. A reliable within-experiment response is achieved only with a minimal variation of the factor. Thus for each factor, the within-experiment variation was calculated and the experiments presenting the 25% lowest variations were not included in the GLM. The influence of qualitative factors on the model parameters was tested with a one-way ANOVA.

In the second step of the analysis, the significant interfering factors were included individually in equation 2. Quantitative factors were tested as additional covariable, either in substitution of the experiment effect (equation [4]) or in addition to the experiment effect (equation [4']):

$$CH_4 = \alpha + \beta \times protozoa + \gamma \times factor + e \qquad [4]$$

$$CH_4 = \alpha + \alpha_i + \beta \times protozoa + \beta_i \times protozoa + \gamma \times factor + \gamma_i \times factor + e \qquad [4']$$

where α, $\alpha_i$, β, $\beta_i$, and e were as defined in equation 1, γ= the linear term for the factor and $\gamma_i$ = the fixed effect of the experiment *i* on the factor slope. This approach allows the identification of factors able to replace the experiment effect while explaining a part of the variability between experiments not explained by the model. Qualitative factors were added as fixed effects to the equation 2 with the experiment effect nested within the factor:

$$CH_4 = \alpha + \alpha_i\,(factor) + \beta \times protozoa + factor + factor \times protozoa + e \quad [5]$$

where α, β and e were as defined in equation 1 and $\alpha_i$ = the fixed effect of the experiment *i* (nested within the qualitative factor) on the overall intercept α. In a third step, significant interfering quantitative factors were included simultaneously in equation 2 to rank them in terms of how much they contributed to the relationship between $CH_4$ and protozoa.

At each step of the meta-analysis process, graphical observations were made to check the coherence of relationships, and to identify obviously abnormal values. All statistical analyses were carried out using Minitab, Version 16. Statistical significance was considered at $P \leq 0.05$ and a trend was declared at $P < 0.1$.

## Results

*Description of the meta-design*

A summary of the main database parameters is given in Table 1. Information is presented separately for large and small ruminants, represented by 37 experiments with dairy and beef cattle and 39 experiments with sheep and goats. No statistical difference was observed between animal species for $CH_4$ emission (g/kg DMI, $P$=0.707; g/kg LW, $P$=0.207), intake level (g DMI/day per kg BW, $P$=0.492), gross energy of the diet (MJ/kg DM, $P$=0.452) or diet CP and OM content (g/kg DM, $P$=0.103 and $P$=0.645, respectively). In contrast, small ruminants had a more fibrous diet with a higher NDF content (g/kg DM, $P$<0.001) and a lower diet OM digestibility (%, $P$=0.001), inducing a higher proportion in the rumen of C2 and lower proportions of C3 and C4 (mol/100mol, $P$<0.001) than in large ruminants. Rumen protozoa concentration ($\log_{10}$ cells/ml) tended to be lower in small than in large ruminants ($P$=0.075).

The $CH_4$ emission tended to be higher when expressed in g/kg digestible OM intake ($P$=0.074), and lower when expressed as a percentage of gross energy intake ($P$=0.097), in small compared to large ruminants. On these reduced datasets presenting measurements of OM digestibility or gross energy intake, $CH_4$ emission expressed in g/kg DMI did not differ between small and large ruminants ($P$=0.899 and $P$=0.481, respectively).

**Table 1** Description of the complete database: methane emission, intake, diet composition and rumen parameters in large and small ruminants

| | Large ruminants | | | | | Small ruminants | | | | | Species effect *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nt | Mean | s.d. | Min | Max | Nt | Mean | s.d. | Min | Max | |
| Methane emission (g/kg DMI) | 96 | 18.7 | 6.4 | 2.4 | 36.3 | 115 | 19.0 | 5.7 | 7.9 | 40.5 | 0.707 |
| Methane emission (g/kg DOMI) | 49 | 27.9 | 10.2 | 3.7 | 51.9 | 72 | 30.7 | 6.9 | 13.0 | 50.9 | 0.074 |
| Methane emission (g/kg LW) | 73 | 0.44 | 0.21 | 0.09 | 1.17 | 104 | 0.48 | 0.23 | 0.10 | 1.39 | 0.207 |
| Methane emission (% of GE intake) | 78 | 5.99 | 1.68 | 2.30 | 10.80 | 67 | 5.50 | 1.86 | 2.36 | 10.41 | 0.097 |
| Intake (g DMI/day per kg BW) | 77 | 24.9 | 9.3 | 11.5 | 43.2 | 100 | 25.9 | 9.6 | 11.5 | 46.4 | 0.492 |
| Dietary composition (g/kg DM) | | | | | | | | | | | |
| OM | 69 | 916.0 | 30.0 | 800.0 | 966.0 | 62 | 913.4 | 33.3 | 804.0 | 949.0 | 0.645 |
| NDF | 77 | 367.0 | 89.4 | 169.0 | 671.0 | 97 | 441.1 | 83.4 | 239.0 | 678.0 | <0.001 |
| Starch | 33 | 227.9 | 129.0 | 22.3 | 472.0 | 6 | 224.0 | 44.2 | 158.0 | 253.0 | 0.943 |
| CP | 81 | 155.3 | 33.0 | 59.0 | 230.0 | 101 | 146.6 | 37.2 | 25.1 | 256.0 | 0.103 |
| Gross energy (MJ/kg DM) | 44 | 18.4 | 1.2 | 16.7 | 21.7 | 46 | 18.5 | 1.0 | 16.4 | 20.0 | 0.578 |
| Concentrate: Forage (%) | 92 | 46.2 | 16.6 | 0.0 | 90.0 | 117 | 23.5 | 26.0 | 0.0 | 83.0 | <0.001 |
| OM total tract digestibility (%) | 59 | 69.2 | 5.5 | 52.0 | 83.0 | 68 | 64.1 | 10.6 | 39.9 | 83.3 | 0.001 |
| Rumen parameters | | | | | | | | | | | |
| Protozoa ($log_{10}$ cells/ml) | 100 | 5.58 | 0.80 | 0.00 | 6.80 | 107 | 5.22 | 1.86 | 0.00 | 7.31 | 0.075 |
| Total VFA (mmol/l) | 85 | 108.0 | 25.0 | 44.7 | 165.3 | 112 | 90.9 | 31.6 | 22.9 | 171.1 | <0.001 |
| C2 (mol/100mol) | 89 | 62.1 | 4.8 | 48.2 | 74.3 | 112 | 69.5 | 4.4 | 60.3 | 79.1 | <0.001 |
| C3 (mol/100mol) | 89 | 21.8 | 4.2 | 12.2 | 36.0 | 112 | 18.4 | 4.1 | 9.5 | 27.5 | <0.001 |
| C4 (mol/100mol) | 89 | 11.5 | 2.0 | 6.7 | 16.1 | 112 | 8.9 | 2.3 | 5.4 | 16.0 | <0.001 |
| C2/C3 | 89 | 2.99 | 0.79 | 1.34 | 6.07 | 112 | 4.02 | 1.20 | 2.34 | 8.22 | <0.001 |
| (C2+C4)/C3 | 89 | 3.54 | 0.91 | 1.54 | 6.89 | 112 | 4.54 | 1.35 | 2.54 | 9.35 | <0.001 |
| pH | 81 | 6.40 | 0.40 | 5.06 | 7.33 | 92 | 6.60 | 0.33 | 5.66 | 7.16 | <0.001 |

Min = Minimum; Max = Maximum; Nt = number of treatments; DMI = dry matter intake; LW = live weight; GE = gross energy; DOMI = digestible organic matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

To measure $CH_4$ emission, 31 experiments (Nt=88) used the $SF_6$ tracer technique and 45 experiments (Nt=131) used open or closed chambers. Protozoa concentration was determined from rumen samples taken before feeding (Nexp=19, Nt=64), after feeding (Nexp=34, Nt=89) and both before and after feeding (Nexp=17, Nt=47). This information was unclear or not reported in six experiments (Nt=19). To determine protozoa concentrations, counting chambers were used in 70 experiments (Nt=201) and six experiments (Nt=18) used qPCR.

Information on $CH_4$ emission (g/kg DMI) and protozoa concentration ($log_{10}$ cells/ml) was collected for 70 experiments (Nt=198). The distribution of these experiments according to their variation in $CH_4$ or protozoa is presented in Table 2.

**Table 2** Number of experiments without or with significant within-experiment variation of protozoa concentration ($log_{10}$ cells/ml) or methane emission (g/kg dry matter intake)

| | No protozoa variation | | Protozoa variation | |
|---|---|---|---|---|
| | No $CH_4$ variation | $CH_4$ variation | No $CH_4$ variation | $CH_4$ variation |
| Biotechnology | | | | |
| Defaunation | 2 | 0 | 3 | 2 |
| Pro/Prebiotics, Microbial products | 2 | 3 | 1 | 0 |
| Additives | | | | |
| Chemicals | 0 | 2 | 0 | 0 |
| Organic acids | 2 | 2 | 0 | 0 |
| Plant extracts | | | | |
| Tannins | 1 | 0 | 2 | 2 |
| Saponins | 5 | 1 | 2 | 0 |
| Essentials oil | 1 | 3 | 0 | 1 |
| Feed components | | | | |
| Lipids | 1 | 4 | 1 | 10 |
| Forages | 5 | 0 | 1 | 1 |
| Concentrates | 0 | 0 | 1 | 1 |
| Association | 0 | 0 | 3 | 5 |

The chi square tests showed that no variation in protozoa was mostly observed in experiments from the "additives" strategy, whereas all the experiments in the "association" strategy reported variation in protozoa ($P$=0.004). Conversely, if the effect on protozoa was not considered, no specific strategy affected $CH_4$ emission ($P$=0.376). Looking simultaneously at their effects on protozoa concentration and/or $CH_4$ emission, strategies were statistically related to specific effects on these parameters ($P$=0.032). No variation in either protozoa or $CH_4$ was observed in 19 experiments, in particular those testing different forages. Conversely, 15 experiments reported a variation in $CH_4$ with no variation in protozoa, mostly experiments

testing chemicals or essential oils. Fourteen experiments reported a reduction of protozoa concentration with no change in $CH_4$ emission. A reduction of protozoa concentration was associated with a reduction of $CH_4$ emission in 22 experiments. Experiments testing tannins and lipids were the most numerous in this last group.

Table 3 reports the correlations between rumen protozoa concentration and quantitative factors. With a global analysis approach, rumen protozoa were negatively correlated to OM and CP total tract digestibility ($P$=0.001), rumen total VFA concentration ($P$=0.001), C3 proportion ($P$<0.001) and bacteria concentration ($P$=0.005). Using the same approach, rumen protozoa concentration was positively correlated to rumen pH ($P$=0.019), proportion of C2 ($P$<0.001) and the ratios C2/C3 and (C2+C4)/C3 ($P$<0.001). Similar trends were observed with the between-experiment analysis approach, except for rumen pH and bacteria which were no longer correlated to protozoa. With the within-experiment approach, intake, NDF digestibility, rumen proportion of C2 and the ratios C2/C3 and (C2+C4)/C3 were positively correlated with protozoa ($P$<0.001, $P$=0.018, $P$=0.009, $P$=0.047 and $P$=0.039, respectively) whereas rumen proportion of C3 was again negatively correlated to this parameter (P=0.003). In none of these approaches were rumen proportion of C4 and number of methanogens correlated to protozoa. The rumen protozoa were significantly affected by animal species, $CH_4$ mitigation strategy and rumen sampling time ($P$=0.027, $P$=0.031, $P$=0.006, respectively; data not shown) and a tendency was observed with the method of distribution (dose-response, source, form) of the additive ($P$=0.061; data not shown).

Table 4 reports the correlations between $CH_4$ emission and quantitative factors. With a global approach, $CH_4$ emissions were negatively correlated with intake ($P$=0.016), C3 proportion ($P$<0.001) and rumen methanogens ($P$=0.012) and positively correlated with OM and NDF digestibility ($P$<0.001 and $P$=0.002), C2 and C4 proportions ($P$=0.012 and $P$<0.001), C2/C3 and (C2+C4)/C3 ratios ($P$=0.007 and $P$=0.001), rumen pH ($P$<0.001) and bacteria concentration ($P$=0.017). With the between-experiment approach, OM digestibility, C4 proportion and rumen pH were also positively correlated with $CH_4$ ($P$=0.008, $P$=0.030 and $P$=0.013, respectively) and C3 proportion and methanogen concentration were negatively correlated with $CH_4$ ($P$=0.009 and $P$=0.016). The within-experiment approach yielded the same information as the between-experiment approach, except that C2 proportion was positively correlated with $CH_4$ ($P$<0.001) unlike C4 proportion ($P$=0.169). Methane emission was not significantly affected by animal species, $CH_4$ mitigation strategy, $CH_4$ method of measurement or the method of distribution of the additive (dose-response, source, form) ($P$=0.131, $P$=0.431, $P$=0.084, $P$=0.331, respectively; data not shown).

**Table 3** Global correlation, between and within-experiment (equation 1) relationship between rumen protozoa concentration and quantitative factors

| | Rumen protozoa concentration ($log_{10}$ cells/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Global | | | Between experiment | | | Within-experiment | | | |
| Quantitative factors | Nt | r | *P*-value | Nexp | r | *P*-value | Nexp | Nt | Slope | *P*-value |
| Intake (g DMI/day per kg BW) | 151 | -0.068 | 0.405 | 55 | -0.079 | 0.564 | 27 | 83 | 2.618 | <0.001 |
| Total tract digestibility (%) | | | | | | | | | | |
| OM | 125 | -0.299 | 0.001 | 43 | -0.326 | 0.033 | 21 | 68 | 1.254 | 0.382 |
| NDF | 125 | -0.032 | 0.725 | 45 | -0.089 | 0.563 | 18 | 57 | 4.305 | 0.018 |
| Starch | 31 | -0.221 | 0.233 | 10 | -0.253 | 0.480 | 4 | 13 | -0.537 | 0.355 |
| CP | 71 | -0.495 | <0.001 | 28 | -0.561 | 0.002 | 11 | 32 | -0.009 | 0.997 |
| Rumen parameters | | | | | | | | | | |
| Total VFA (mmol/l) | 164 | -0.249 | 0.001 | 57 | -0.243 | 0.068 | 25 | 80 | -0.372 | 0.921 |
| C2 (mol/100mol) | 168 | 0.365 | <0.001 | 59 | 0.361 | 0.005 | 26 | 82 | 2.310 | 0.009 |
| C3 (mol/100mol) | 168 | -0.435 | <0.001 | 59 | -0.452 | <0.001 | 26 | 82 | -2.432 | 0.003 |
| C4 (mol/100mol) | 168 | -0.035 | 0.656 | 59 | -0.062 | 0.643 | 26 | 82 | 0.665 | 0.159 |
| C2/C3 | 168 | 0.462 | <0.001 | 59 | 0.528 | <0.001 | 26 | 82 | 0.426 | 0.047 |
| (C2+C4)/C3 | 168 | 0.460 | <0.001 | 59 | 0.525 | <0.001 | 26 | 82 | 0.512 | 0.039 |
| pH | 154 | 0.188 | 0.019 | 54 | 0.217 | 0.116 | 23 | 74 | 0.116 | 0.065 |
| Methanogens (cells/ml) | 28 | 0.117 | 0.555 | 12 | -0.010 | 0.975 | 3 | 8 | 4.090 | 0.717 |
| Bacteria (cells/ml) | 67 | -0.340 | 0.005 | 22 | -0.320 | 0.146 | 13 | 45 | -6.200 | 0.725 |

Nexp = number of experiments; Nt = number of treatments; DMI = dry matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

**Table 4** Global correlation, between and within-experiment (equation 1') relationship between methane emission and quantitative factors

| | Methane emission (g/kg DMI) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Global | | | Between experiment | | | Within-experiment | | | |
| Quantitative factors | Nt | r | *P*-value | Nexp | r | *P*-value | Nexp | Nt | Slope | *P*-value |
| Intake (g DMI/day per kg BW) | 159 | -0.191 | 0.016 | 59 | -0.196 | 0.138 | 34 | 99 | 0.038 | 0.382 |
| Total tract digestibility (%) | | | | | | | | | | |
| OM | 121 | 0.369 | <0.001 | 42 | 0.404 | 0.008 | 21 | 69 | 0.292 | 0.002 |
| NDF | 121 | 0.278 | 0.002 | 44 | 0.269 | 0.077 | 22 | 65 | 0.356 | 0.001 |
| Starch | 31 | 0.082 | 0.661 | 10 | 0.019 | 0.958 | 7 | 25 | 0.053 | 0.809 |
| CP | 67 | 0.085 | 0.492 | 27 | 0.162 | 0.420 | 12 | 32 | -0.176 | 0.285 |
| Rumen parameters | | | | | | | | | | |
| Total VFA (mmol/l) | 172 | 0.018 | 0.819 | 61 | 0.008 | 0.951 | 33 | 102 | 0.046 | 0.879 |
| C2 (mol/100mol) | 176 | 0.188 | 0.012 | 63 | 0.174 | 0.171 | 35 | 106 | 0.280 | <0.001 |
| C3 (mol/100mol) | 176 | -0.333 | <0.001 | 63 | -0.328 | 0.009 | 35 | 106 | -0.312 | <0.001 |
| C4 (mol/100mol) | 176 | 0.269 | <0.001 | 63 | 0.274 | 0.030 | 35 | 106 | 0.049 | 0.169 |
| C2/C3 | 176 | 0.204 | 0.007 | 63 | 0.188 | 0.139 | 35 | 106 | 0.075 | <0.001 |
| (C2+C4)/C3 | 176 | 0.238 | 0.001 | 63 | 0.225 | 0.077 | 35 | 106 | 0.087 | <0.001 |
| pH | 160 | 0.293 | <0.001 | 57 | 0.328 | 0.013 | 28 | 84 | 0.004 | 0.399 |
| Methanogens (cells/ml) | 28 | -0.468 | 0.012 | 12 | -0.673 | 0.016 | 9 | 22 | 0.262 | 0.097 |
| Bacteria (cells/ml) | 67 | 0.291 | 0.017 | 22 | 0.373 | 0.088 | 10 | 34 | -0.770 | 0.910 |

Nexp = number of experiments; Nt = number of treatments; DMI = dry matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

*Effects of a variation of rumen protozoa concentration on $CH_4$ emission*

The within-experiment relationship between rumen protozoa concentration and $CH_4$ emission is presented in Figure 1. When protozoa concentration ranged between 4.5 and 7.3 $\log_{10}$ cells/ml (0.3 and $206\times10^5$ cells/ml), the response law relating $CH_4$ emission (g/kg DMI) to rumen protozoa concentration ($\log_{10}$ cells/ml) was linear (equation 2):

$$CH_4 = -30.74 \text{ (s.e. } 5.09)^{***} + 8.14 \text{ (s.e. } 0.85)^{***} \times \text{protozoa}$$

Where Nt = 91, Nexp = 28, residual mean square error (r.m.s.e.) = 1.94, $R^2$ = 0.93, adjusted $R^2$ = 0.90 and Nout = 0.

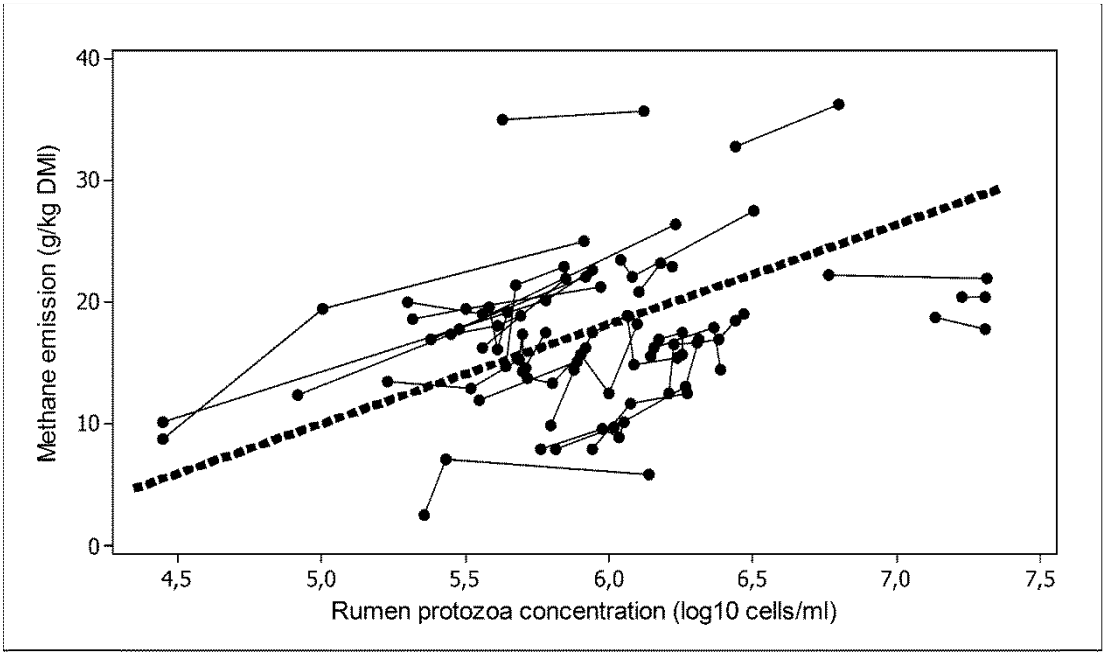


**Figure 1** Relationship between methane emission and rumen protozoa concentration (raw data). The black dashed line represents the average within-experiment relationship (equation 2).

*Effects of interfering factors for the response law relating $CH_4$ to protozoa*

Table 5 presents the correlations between slopes and LSMeans of experiments from equation 2 with quantitative factors. One experiment presenting a very high slope value had to be excluded to get a normal distribution of slopes ($P$=0.210) and LSMeans ($P$=0.141). The digestibility of OM and CP and the rumen proportion of C4 were positively correlated to LSMeans ($P$=0.013, $P$<0.001 and $P$=0.017, respectively) and slopes were correlated with intake ($P$=0.018) and CP digestibility ($P$=0.016). No other significant correlation was observed.

**Table 5** Correlations between slopes and LSMeans of experiments from equation 2 with quantitative factors

| Quantitative factors | Nexp | Slope | | LSMeans | |
|---|---|---|---|---|---|
| | | r | *P*-value | r | *P*-value |
| Intake (g DMI/day per kg BW) | 26 | -0.460 | 0.018 | -0.320 | 0.111 |
| Total tract digestibility (%) | | | | | |
| OM | 20 | 0.113 | 0.635 | 0.544 | 0.013 |
| NDF | 17 | 0.151 | 0.564 | -0.080 | 0.759 |
| Starch | 4 | -0.126 | 0.874 | 0.657 | 0.343 |
| CP | 10 | 0.731 | 0.016 | 0.911 | <0.001 |
| Rumen parameters | | | | | |
| Total VFA (mmol/l) | 25 | -0.369 | 0.069 | 0.062 | 0.770 |
| C2 (mol/100mol) | 25 | -0.153 | 0.465 | -0.122 | 0.560 |
| C3 (mol/100mol) | 25 | 0.192 | 0.357 | 0.003 | 0.988 |
| C4 (mol/100mol) | 25 | 0.021 | 0.919 | 0.474 | 0.017 |
| C2/C3 | 25 | -0.257 | 0.216 | -0.196 | 0.348 |
| (C2+C4)/C3 | 25 | -0.259 | 0.211 | -0.158 | 0.450 |
| pH | 22 | -0.096 | 0.670 | -0.083 | 0.712 |
| Methanogens (cells/ml) | 3 | 0.890 | 0.301 | -0.753 | 0.458 |
| Bacteria (cells/ml) | 13 | 0.161 | 0.600 | -0.183 | 0.550 |

Nexp = number of experiments; LSMeans = least square means; DMI = dry matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

Table 6 gives the within-experiment correlation between quantitative factors and residuals determined from equation 2 for all the experiments in the database. The distribution of calculated residuals did not significantly differ from normality ($P$=0.054). They were positively correlated to rumen proportion of C2 ($P$=0.008) and the ratios C2/C3 and (C2+C4)/C3 ($P$<0.001) and negatively correlated to rumen proportion of C3 ($P$=0.013). No qualitative factors influenced slopes or LSMeans but residuals were influenced by method of $CH_4$ measurement, $CH_4$ mitigation strategy, distribution of additive, animal species and rumen sampling time ($P$=0.003, $P$=0.021, $P$=0.003, $P$=0.018 and $P$=0.006, respectively; data not shown).

**Table 6** Within-experiment relationship between the residuals (observed $CH_4$ minus predicted $CH_4$ with equation 2) and quantitative factors (equation 3)

| Quantitative factors | Var | Nexp | Nt | Nout | Intercept (s.e.) | *P*-value | Slope (s.e.) | *P*-value | r.m.s.e. | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Intake (g DMI/day per kg BW) | 0.3 | 41 | 119 | 2 | 13.5 (5.60) | 0.019 | -0.402 (0.213) | 0.062 | 2.970 | 0.88 |
| Total tract digestibility (%) | | | | | | | | | | |
| OM | 1.8 | 32 | 94 | 3 | -10.7 (7.49) | 0.157 | 0.202 (0.113) | 0.078 | 3.118 | 0.86 |
| NDF | 2.3 | 31 | 92 | 3 | -1.2 (4.73) | 0.806 | 0.096 (0.085) | 0.265 | 3.206 | 0.80 |
| Starch | 0.3 | 8 | 27 | 0 | -9.5 (31.75) | 0.769 | 0.123 (0.337) | 0.719 | 4.205 | 0.68 |
| CP | 1.2 | 20 | 50 | 2 | 5.2 (5.44) | 0.350 | -0.029 (0.090) | 0.752 | 2.492 | 0.91 |
| Rumen parameters | | | | | | | | | | |
| Total VFA (mmol/l) | 4.0 | 43 | 128 | 4 | 2.3 (3.48) | 0.514 | 0.009 (0.035) | 0.800 | 3.131 | 0.88 |
| C2 (mol/100mol) | 1.1 | 43 | 126 | 1 | -23.2 (9.97) | 0.023 | 0.420 (0.154) | 0.008 | 3.183 | 0.87 |
| C3 (mol/100mol) | 1.1 | 43 | 125 | 1 | 12.4 (3.65) | 0.001 | -0.436 (0.172) | 0.013 | 3.325 | 0.84 |
| C4 (mol/100mol) | 0.7 | 46 | 135 | 3 | 3.4 (2.96) | 0.257 | 0.020 (0.283) | 0.945 | 3.147 | 0.86 |
| C2/C3 | 0.2 | 43 | 123 | 1 | -5.3 (2.37) | 0.028 | 2.544 (0.667) | <0.001 | 3.086 | 0.88 |
| (C2+C4)/C3 | 0.3 | 42 | 121 | 1 | -5.5 (2.44) | 0.027 | 2.216 (0.603) | <0.001 | 3.164 | 0.87 |
| pH | 0.1 | 40 | 119 | 3 | 6.6 (17.62) | 0.709 | -0.466 (2.717) | 0.864 | 3.219 | 0.87 |
| Methanogens (cells/ml) | 0.2 | 10 | 23 | 0 | 1.1 (1.92) | 0.582 | 0.756 (0.370) | 0.064 | 3.435 | 0.81 |
| Bacteria (cells/ml) | 1.0 | 14 | 48 | 1 | 0.7 (0.93) | 0.485 | -0.002 (0.004) | 0.647 | 2.777 | 0.92 |

Var = minimum within-experiment variation level of the tested factor; Nexp = number of experiments; Nt = number of treatments; Nout = number of outliers; s.e. = standard error; r.m.s.e. = residual mean square error; DMI = dry matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

**Table 7** Relationship between methane emission (g/kg dry matter intake) and rumen protozoa concentration ($log_{10}$ cells/ml) with quantitative factors in addition (equation 4') or in substitution (equation 4) of the experiment effect

| | | | | | | Protozoa | | Factor | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Quantitative factors | Nexp | Nt | Nout | Intercept (s.e.) | P-value | Slope (s.e.) | P-value | Slope (s.e.) | P-value | r.m.s.e | $R^2$ |
| Equation 2 | 28 | 91 | 0 | -30.7 (5.1) | <0.001 | 8.14 (0.85) | <0.001 | - | - | 1.94 | 0.93 |
| With experiment effect (equation 4') | | | | | | | | | | | |
| Intake (g DMI/day per kg BW) | 24 | 74 | 0 | -28.3 (6.5) | <0.001 | 8.51 (0.99) | <0.001 | -0.202 (0.223) | 0.371 | 2.02 | 0.94 |
| OM digestibility (%) | 18 | 59 | 0 | -38.7 (7.8) | 0.001 | 8.14 (0.87) | <0.001 | 0.101 (0.098) | 0.309 | 1.82 | 0.92 |
| CP digestibility (%) | 10 | 30 | 0 | -15.0 (9.5) | 0.131 | 8.12 (1.12) | <0.001 | -0.273 (0.108) | 0.020 | 1.61 | 0.89 |
| C2 (mol/100mol) | 23 | 73 | 0 | -42.9 (10.3) | <0.001 | 5.97 (0.93) | <0.001 | 0.376 (0.164) | 0.027 | 1.68 | 0.95 |
| C3 (mol/100mol) | 23 | 73 | 1 | -0.7 (7.2) | 0.918 | 4.98 (0.90) | <0.001 | -0.578 (0.142) | <0.001 | 1.53 | 0.96 |
| C4 (mol/100mol) | 23 | 73 | 0 | -24.0 (5.3) | <0.001 | 6.13 (0.91) | <0.001 | 0.527 (0.226) | 0.024 | 1.68 | 0.95 |
| C2/C3 | 23 | 73 | 1 | -24.3 (4.8) | <0.001 | 5.50 (0.86) | <0.001 | 2.437 (0.619) | <0.001 | 1.54 | 0.96 |
| (C2+C4)/C3 | 23 | 73 | 1 | -23.6 (4.8) | <0.001 | 5.39 (0.85) | <0.001 | 2.110 (0.515) | <0.001 | 1.53 | 0.96 |
| Without experiment effect (equation 4) | | | | | | | | | | | |
| Intake (g DMI/day per kg BW) | - | 74 | 1 | -7.1 (8.1) | 0.385 | 5.01 (1.38) | 0.001 | -0.229 (0.099) | 0.023 | 6.11 | 0.18 |
| OM digestibility (%) | - | 59 | 1 | -30.4 (8.0) | <0.001 | 5.16 (1.00) | <0.001 | 0.253 (0.059) | <0.001 | 4.22 | 0.38 |
| CP digestibility (%) | - | 30 | 0 | -30.6 (11.7) | 0.015 | 5.67 (1.27) | <0.001 | 0.247 (0.076) | 0.003 | 3.00 | 0.43 |
| C2 (mol/100mol) | - | 73 | 1 | -8.2 (9.0) | 0.362 | 4.19 (1.54) | 0.008 | 0.009 (0.115) | 0.937 | 6.07 | 0.12 |
| C3 (mol/100mol) | - | 73 | 1 | 1.04 (11.2) | 0.927 | 3.33 (1.57) | 0.038 | -0.182 (0.156) | 0.245 | 6.01 | 0.14 |
| C4 (mol/100mol) | - | 73 | 2 | -21.9 (8.2) | 0.010 | 4.77 (1.25) | <0.001 | 1.093 (0.274) | <0.001 | 5.48 | 0.28 |
| C2/C3 | - | 73 | 1 | -9.2 (8.8) | 0.303 | 4.58 (1.65) | 0.007 | -0.214 (0.570) | 0.709 | 6.06 | 0.12 |
| (C2+C4)/C3 | - | 73 | 1 | -8.0 (8.8) | 0.368 | 4.25 (1.65) | 0.012 | -0.002 (0.530) | 0.996 | 6.07 | 0.12 |

Nexp = number of experiments; Nt = number of treatments; Nout = number of outliers; s.e. = standard error; r.m.s.e. = residual mean square error; DMI = dry matter intake; OM = organic matter; C2 = acetate; C3 = propionate; C4 = butyrate.

None of the qualitative factors appeared significant when included in the model with the experiment effect nested within the factor (equation 5, data not shown). Table 7 shows the response law relating $CH_4$ to protozoa with significant quantitative factors added to or substituted for the experiment effect. Added to experiment effect, CP digestibility and C3 proportion were negatively correlated to $CH_4$ emission ($P$=0.020 and $P$<0.001), whereas C2 and C4 proportions and the ratios C2/C3 and (C2+C4)/C3 were positively correlated to $CH_4$ emission ($P$=0.027, $P$=0.024, $P$<0.001 and $P$<0.001, respectively). Substituted for experiment effect, OM and CP digestibility and rumen C4 proportion were positively correlated to $CH_4$ emission ($P$<0.001, $P$=0.003 and $P$<0.001, respectively), whereas intake was negatively correlated to $CH_4$ emission ($P$=0.023). When simultaneously including these four quantitative factors in equation 2 together with protozoa concentration (data not shown), protozoa concentration ($P$=0.028) and C4 proportion (P=0.018) explained 39% and 48% of the variability, experiment effect excluded. Intake and digestibility of OM and CP digestibility were not significant.

**Discussion**

The database was well-balanced for animal species, with almost the same number of treatments between small and large ruminants. A confounding effect between diet composition and animal species was noteworthy, with large ruminants having a diet richer in energy than small ruminants. This led to differences in rumen fermentation profiles, with lower proportion of C2 and higher proportion of C3 in large ruminants. However, $CH_4$ emission (expressed in g/kg DMI or g/kg LW) and protozoa concentration ($log_{10}$ cells/ml) were homogeneously distributed between animal species. Consequently, it appears unlikely that any potential animal species effect would be revealed in further analyses.

*Influence of $CH_4$ mitigation strategy on $CH_4$ and protozoa*

Although the database was not built to evaluate mitigation strategies for their effect on $CH_4$ emission and rumen protozoa, the chi square tests highlighted that most experiments testing lipids or tannins reduced both protozoa concentration and $CH_4$ emission. This information confirmed that a potential mode of action of these compounds on methanogenesis is through protozoal inhibition. These additives may change protozoa membrane permeability, leading to cell lysis (Doreau and Ferlay, 1995; Goel et al., 2005). As reported in a previous review, the effect of these compounds is variable depending on the source, the mode and the length of administration (Popova et al., 2011). This could explain the variability of the effects of these

additives on protozoa concentration. As an example, lipid effect on protozoa is dependent on the fatty acid profile, with a higher effect of medium chain fatty acids than polyunsaturated ones, which was confirmed by our data: lauric acid tended to reduce protozoa more markedly than polyunsaturated fatty acids (Jordan et al., 2006).

Defaunation studies did not necessarily observe a reduction of $CH_4$ emission. Difference in diets may explain this variable effect as removal of protozoa has a more marked effect on methanogenesis with high concentrate diets (Hegarty, 1999). However, this effect was not clearly seen in our database, as two out of four experiments reporting no variation in $CH_4$ emission after defaunation used a diet with 83% of concentrate. Conversely, in the two experiments showing a reduction of $CH_4$ emission after defaunation, animals were fed a diet with more than 60% of concentrate.

Chemicals, essential oils and organic acids were identified as methanogenesis reducers without affecting protozoa. Two different mechanisms can be pointed out for these additives. On the one hand, some essential oils are known to directly inhibit growth and activity of methanogens inducing a direct reduction of $CH_4$ emission (Calsamiglia et al., 2007). On the other hand, some chemicals and organic acids divert $H_2$ from methanogenesis to other pathways. For example, nitrate and sulfate are reduced to ammonia and hydrogen sulfide, respectively, with the consumption of four moles of $H_2$ (Ungerfeld and Kohn, 2006). Enhancing C3 synthesis with malate or fumarate, which are precursors of C3, is another way to divert $H_2$ from methanogenesis (Ungerfeld et al., 2007). However, experiments testing organic acids in our database were inconclusive, as already reported in a previous review (Hook et al., 2010). A part of added fumarate may be used for C2 production, balancing the effect on C3 production (Ungerfeld et al., 2007).

Finally, in our dataset, forage modification, addition of probiotics, prebiotics or exogenous microbial products had a weak influence on protozoa concentration, while their effect on $CH_4$ emission was variable. The mechanisms of action of these additives on $CH_4$ emission remain to be clarified. Probiotics and prebiotics may either enhance specific microbial groups able to use excess $H_2$ for C3 synthesis, or stimulate microbial growth leading to a higher $H_2$ consumption for microbial biomass synthesis (Jeyanathan et al., 2014). However, in one experiment testing probiotics, $CH_4$ yield was reduced by 25% with no changes in ruminal fermentation and protozoa (Lettat, 2012).

*Effects of a variation in protozoa concentration on $CH_4$ emission*

To our knowledge, only one publication has established a quantitative relationship between numbers of protozoa and $CH_4$ emission (Morgavi et al., 2010). In that work using a dataset of 21 experiments, the number of protozoa explained 47% of the variability in $CH_4$ emission (r.m.s.e. = 3.25). Methane was reduced by 1g $CH_4$/kg DMI by every decrease of 0.12 $log_{10}$ protozoa cells/ml. In agreement with these findings, we showed that rumen protozoa concentration explained 93% of the variability in $CH_4$ emission, and that a reduction of 0.12 $log_{10}$ protozoa cells/ml induced a reduction of 1g $CH_4$/kg DMI (r.m.s.e. = 1.94). Our analysis is more reliable than the previous work as it included seven additional experiments and presented a lower r.m.s.e. In addition, our approach distinguished between intra and inter-experiment effects, and focused more specifically on experiments with a significant within-experiment variation of protozoa concentration. The equation 2 can be used to quantify with a good accuracy the impact of changes in protozoa concentration (in the range 4.5-7.3 $log_{10}$ cells/ml) on $CH_4$ emission in the wide diversity of intake level and diet composition defined by the meta-design. However, the significant experiment effect implies that this equation cannot accurately estimate the absolute $CH_4$ emission from a measured protozoa concentration. Consequently, the study of interfering factors is required.

*Interfering factors for the response law relating $CH_4$ to protozoa*

One aim of this study was to improve our understanding of the relationship between $CH_4$ and protozoa by testing different quantitative and qualitative potential interfering factors. A reliable interfering factor can be accepted if its inclusion into the response law does not lead to a large variation in the initial equation slope (protozoa linear term). When including the experiment effect, the slopes associated with CP digestibility, VFA proportions and the ratios C2/C3 and (C2+C4)/C3 were significant, but the r.m.s.e. of the overall equations were only slightly improved. However, the change in the mean slope (or its s.e.) associated with protozoa demonstrated confounding effects between quantitative interfering factors and experiment effect. A positive relationship between the C2/C3 ratio and $CH_4$ emission has already been quantified by a meta-analysis approach (Sauvant et al., 2011). With the present database, a similar relationship was observed ($P<0.05$, data not shown), and the residuals of this relationship were evidenced to be positively correlated to rumen protozoa ($P<0.001$, data not shown).

When substituting for the experiment effect, intake, OM and CP digestibility, and rumen proportion of C4 significantly influenced the response law relating $CH_4$ to protozoa, but

strongly modified the slope associated with protozoa, and markedly increased the r.m.s.e. This result shows that taking into account experimental effects provides the most precise estimate of the influence of protozoa on $CH_4$ production.

When simultaneously adding intake, OM and CP digestibility and rumen proportion of C4 in equation 2, C4 proportion was the main interfering quantitative factor, with a strong contribution to the explained variability. It is known that protozoa preferentially ferment OM to C4 rather than to C2 or C3 (Williams and Coleman, 1992; Brossard et al., 2004). Surprisingly, in our database, we did not find any significant relationship between protozoa and C4, showing that C4 concentration cannot be considered as a direct indicator of rumen protozoa activity. Other microbial populations may be responsible for C4 production, such as *Butyrivibrio fibrisolvens* (Stewart et al., 1997). Unfortunately, our database contained limited information about quantity or diversity of other rumen microbes, precluding further analyses.

The response law relating $CH_4$ to protozoa is independent of qualitative factors such as method of $CH_4$ measurement, animal species or $CH_4$ mitigation strategy. No effect of mitigation strategies was observed on the relationship between protozoa and $CH_4$, as only experiments showing a relevant within-experiment variation of protozoa concentration were included in the analysis, which strongly oriented the selection towards experiments testing lipids (nearly half of the eligible experiments).

## Conclusion

By building an exhaustive database from experiments with data for $CH_4$ emission and rumen protozoa concentration on the same groups of animals, we showed that a reduction of protozoa concentration was in most cases indicative of a reduction of $CH_4$ emission. We also quantitatively assessed the effect of a variation in protozoa concentration on $CH_4$ emission. We showed that this relationship was positively influenced by the proportion of butyrate in the rumen.

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# Experimental strategy & materials and methods

**Table 10** Steps and associated experiments conducted during the PhD thesis and justification for selection of animals and dietary treatments

| Steps | Associated experiment (approach) | Animal type<br>Number<br>Experimental design | Dietary treatments | Justification | | Perspective |
|---|---|---|---|---|---|---|
| | | | | Animals | Dietary treatments | |
| 1 & 4 | 1<br>(In vivo) | Non-lactating cows<br>n = 4<br>2 × 2 factorial design | 1/ CON: 50% hay + 50% concentrate<br>2/ NIT: CON + 2.3% nitrate (from calcium nitrate)<br>3/ LIN: CON + 2.6% added lipids (from linseed oil)<br>4/ LIN+NIT: CON + 2.3% nitrate + 1.0% added lipids | Physiologically stable animals | • High-starch diet to favor protozoa<br>• NIT and LIN doses calculated to reach 15-20% $CH_4$ reduction when fed individually and 30-40% reduction when fed in association | Fundamental and mechanistic study |
| 2 | 2<br>(In vivo) | Lactating cows<br>n = 8<br>Randomized block design | 1/ CON: 54% maize silage + 6% hay + 40% concentrate<br>2/ LIN+NIT: CON + 1.8% nitrate (from calcium nitrate) + 3.5% added lipids (from extruded linseed) | Animals farm of interest | • Basal diet close to farm conditions<br>• Lower NIT dose to avoid health issues with producing animals<br>• Extruded linseed chosen as favored in animal feed production (pelleting process is more difficult with oil) | On-farm applicability |
| 3 & 4 | 3<br>(In vivo) | Non-lactating cows<br>n = 4<br>2 × 2 factorial design | 1/ CON: 50% hay + 50% concentrate<br>2/ NIT: CON + 2.3% nitrate (from calcium nitrate)<br>3/ TEA: CON + 0.5% saponin (from tea)<br>4/ TEA+NIT: CON + 2.3% nitrate + 0.5% saponin | Physiologically stable animals | • High-starch diet to favor protozoa<br>• NIT and TEA doses calculated to reach 15-20% $CH_4$ reduction when fed individually and 30-40% reduction when fed in association | Fundamental and mechanistic study |
| 3 | | Lactating cows<br>n = 7<br>2 × 2 crossover design | 1/ CON: 54% maize silage + 6% hay + 40% concentrate<br>2/ TEA: CON + 0.5% saponin (from tea) | Animals farm of interest | • Basal diet close to farm conditions<br>• TEA dose similar to the experiment with non-lactating cows | On-farm applicability |
| 5 | 4<br>(In vitro) | Non-lactating cows<br>n = 2<br>2 repeated incubations | 1/ CON: 50% hay + 50% concentrate<br>2/ CON + 1, 2, 4 or 6 mM nitrate (from ammonium nitrate) | Physiologically stable animals | • Basal diet close to diet fed in experiments 1 and 3 to non-lactating cows<br>• Nitrate doses chosen from literature review | Fundamental and mechanistic study |
| | | Non-lactating cows<br>n = 2<br>2 repeated incubations | 1/ CON: 100% glucose<br>2/ CON + 1, 2, 4 or 6 mM nitrate (from ammonium nitrate) | Physiologically stable animals | • Basal diet chosen to favor microbial biomass synthesis<br>• Nitrate doses chosen from literature review | |

# I. EXPERIMENTAL STRATEGY OF THE PHD THESIS

The literature review highlighted the importance of ruminal $H_2$ pool in methanogenesis. Nowadays, dietary $CH_4$-mitigating strategies aimed at reducing its availability for methanogens *via* a reduction of its production **or** a modification of its utilization. In the meta-analysis, we reported that lipids and plant extracts would be the most pertinent strategies to reduce $H_2$ production *via* a reduction of protozoa, whereas nitrate would be the best user of $H_2$ competing with methanogenesis. However, these strategies have been tested individually to reduce methanogenesis, but no studies reported the $CH_4$-mitigating effect of their association.

We assumed that simultaneous manipulation of $H_2$ production AND utilization allows a more important reduction of $CH_4$ emissions than when acting on a single pathway (production OR utilization). Consequently the originality of our experimental approach consisted in associating lipids or plant extract with nitrate, in order to combine dietary strategies having different mechanisms of action on the rumen $H_2$ pool. Then, this PhD thesis was divided into 5 steps, corresponding to 4 experiments (Table 10), which objectives were:

**Step 1.** 1/ To evaluate the effect of association of feeding strategies acting on $H_2$ production (**lipids from linseed**, toxic effect towards protozoa) and $H_2$ utilization (**nitrate from calcium nitrate**, $H_2$-sink through nitrate reduction to nitrite and ammonia) on $CH_4$ emissions, diet digestibility and N balance of non-lactating cows. 2/ To understand the $CH_4$-mitigating effect of these feeding strategies fed alone or in association by focusing on rumen $H_2$ pool and fermentation parameters.

**Step 2.** 1/ To evaluate the long-term effect of **linseed plus nitrate** on $CH_4$ emissions, lactating performances of dairy cows and animal health (blood metHb, nitrate and nitrite residues in milk and processed milk products). 2/ To check the effect of linseed plus nitrate on total tract digestibility, N balance and rumen fermentation after long-term supplementation.

**Step 3.** 1/ To evaluate the $CH_4$-mitigating effect and associated ruminal mechanisms of another feeding strategy acting on $H_2$ production (**saponin from tea**, toxic effect towards protozoa) fed alone or in association with nitrate to non-lactating cows. 2/ To assess effect of tea saponin on diet digestibility, N balance and lactating performances.

**Step 4.** To understand the effect of tested $CH_4$-mitigating strategies fed alone (linseed, tea saponin, nitrate) or in association (linseed plus nitrate or tea saponin plus nitrate) on the quantity, activity and diversity of rumen microbiota from non-lactating cows.

**Step 5.** 1/ To study the dose response effect of nitrate on *in vitro* production of rumen fermentation end-products such as gas ($CH_4$ and $H_2$), VFA and microbial biomass (estimated from insoluble protein). 2/ To understand the $CH_4$-mitigating mechanisms of nitrate by estimating metabolic $H_2$ distribution between rumen fermentation end-products.

## II. MATERIALS AND METHODS

During this PhD thesis, two new techniques have been developed in the team and will be detailed in the next sections: i) continuous and *in vivo* measurement of enteric $CH_4$ emissions with open chambers; ii) continuous and *in situ* measurement of dissolved $H_2$ concentration in the rumen.

### *2.1. Continuous and in vivo measurement of enteric methane emissions: open chambers*

Quantification of individual $CH_4$ emissions is an essential measurement in this work. Currently, our team has the skills and expertise in the quantification of $CH_4$ emissions using the SF6 tracer technique. However, this method does not give indications about daily kinetics of emissions (Johnson et al., 1994). Inversely, the chamber technique is considered as the reference technique, and has the advantage to continuously quantify $CH_4$ (and $CO_2$) emissions of ruminants, which provides interesting information to explain fermentation pattern (Pinares-Patiño and Waghorn, 2012). Consequently, four open chambers for cattle were built by the team in 2012 and were firstly used during this PhD thesis.

#### 2.1.1. Description of the system and measuring principle

To measure kinetics of enteric $CH_4$ (and $CO_2$) emissions of cattle, our system comprised 3 main components:

1/ **The open chamber** was 2.2 m high, 3.6 m long and 2.1 m wide, giving a volume of 16.6 $m^3$. Floor dimensions gave the animal a 2 $m^2$ movement area, which was close to its stall condition. The chambers were made of steel with transparent polycarbonate walls allowing

sight contact between animals and with the farm personnel. Chambers had front and rear doors, with the front doors used for animal feeding and the rear doors used to enter or milk the animals, or to remove feces and urine collected once daily in a wheeled box.

2/ **The ventilation system** produced an airflow between 500 and 1000 m³/h. There was no automated controller to adjust the airflow to the size and type of animal or to the gas concentrations in chambers. In our experiments, airflow was manually set and averaged 750±50 m³/h (approximately 45 air changes per h) in each chamber. Air entered the chamber through an aperture at the bottom of the rear door (20 cm high, 2.1 m long). The air exited the chamber thanks to the air extractor *via* the exhaust duct situated at the top of the chamber, above the head of the animal. Airflow in the exhaust duct of each chamber was continuously measured (CP300, KIMO, Montpon-Ménestérol, France) and recorded with one data point every 5 min (KT-210-AO, KIMO, Montpon-Ménestérol, France).

3/ **The gas analyzer** (Ultramat 6, Siemens, Karlsruhe, Germany) alternatively measured concentration (ppm) of gases ($CH_4$ and $CO_2$) in the barn (ambient air) and in the four chambers at a 0.1 Hz sample frequency (one data every 10 sec) for 5 min every 25 min. Gas sample from ambient air was taken at the bottom of the rear doors from the four chambers, where entered the airflow. Gas samples from each chamber were taken from the exhaust duct. When entering the analyzer, gas samples were dried with a filter. The analyzer was fitted with a data recording system (Nanodac Invensys, Eurotherm Automation SAS, Dardilly, France).

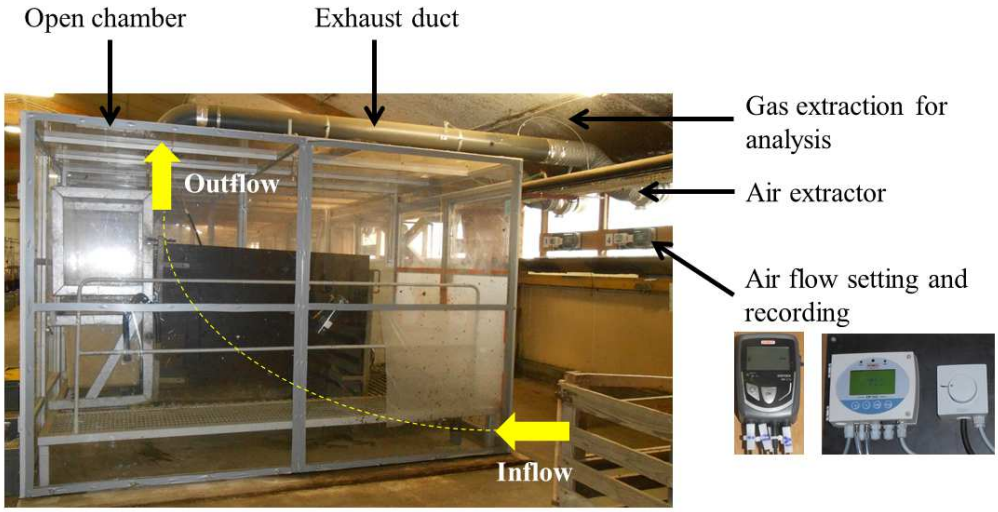


**Figure 15** Description of the system for continuous monitoring of enteric $CH_4$ emissions from cattle. The yellow arrows linked with the dotted line indicate the direction of the air flow within the open chamber, from the inflow to the outflow.

The gas analyzer operated with an infrared (IR) detector, using the principle that some gases are able to absorb specific wavelengths of IR rays (**Figure 16**). A transmitter sent an infrared radiation which was divided into two beams: i) the reference beam which passed through a reference cell with nitrogen gas ($N_2$) resistant to IR rays; ii) the measurement beam which passed though the measurement cell with the gas sample to analyze. As $CH_4$ and $CO_2$ absorb IR radiation ($CH_4$: 3-9 µm; $CO_2$: 14 µm), the concentration of $CH_4$ and $CO_2$ was positively correlated with the amount of absorbed IR rays. Then, the reference and measurement beams arrived in the receiving cell with the detector. They were compared using the reference beam as a baseline, and the amount of exiting IR rays was quantified. According to the calibration curve, the concentration of $CH_4$ and $CO_2$ were finally calculated.

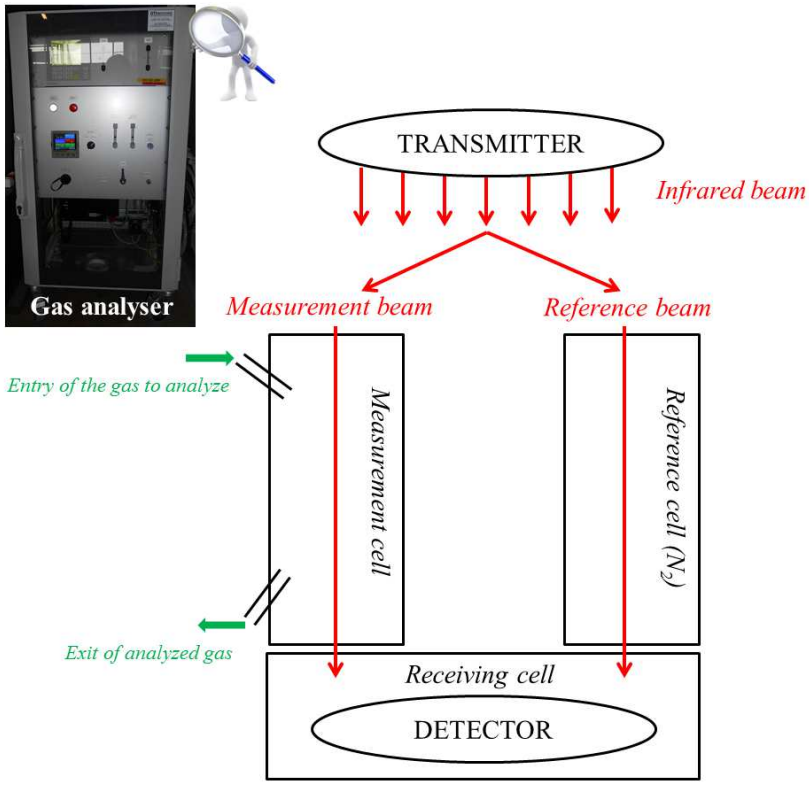


**Figure 16** Functional schematic of the methane and carbon dioxide gas analyzer

2.1.2. System setup and functioning

The week before starting measurement, the gas analyzer was calibrated with a defined gas mixture of $CH_4$ (650 ppm) and $CO_2$ (700 ppm), and with a pure gas ($N_2$) which allowed blank calibration. During the measurement week, airflow and gas data were collected daily, and treated with an home-made Excel macro to calculate $CH_4$ emissions (L/day):

1/ For each chamber and ambient, $CH_4$ data were averaged over the 5-min interval and interpolated by linear regression to get one data point every 5 min.
2/ For each data point and for each chamber, ambient $CH_4$ concentration was subtracted to $CH_4$ concentration of each chamber.
3/ For each chamber, $CH_4$ emissions (L/day) were calculated from $CH_4$ concentration (ppm) and airflow (L/h):

$$CH_4\ (L/day)\ =\ CH_4\ (ppm) \times 10^{-6} \times airflow \times 24$$

Since the gas going into the analyzer was dried, we assumed that to obtain $CH_4$ emissions in the environmental sampling conditions, it was necessary to apply the Wexler equation on airflow data (Pinares-Patiño et al., 2012b). This equation required to get the temperature (T), pressure (P) and relative humidity (RH) in the chamber (exhaust duct) to calculate the volume mixing ratio of water vapor (VMR):

$$VMR = \frac{(a_1 + a_2 \times T + a_3 \times T^2 + a_4 \times T^3 + a_5 \times T^4 + a_6 \times T^5 + a_7 \times T^6) \times RH}{P}$$

With $a_1$, $a_2$, $a_3$, $a_4$, $a_5$, $a_6$ and $a_7$ being the coefficients of water vapor (6.11, 0.44, $1.43 \times 10^{-2}$, $2.65 \times 10^{-4}$, $3.02 \times 10^{-6}$, $2.04 \times 10^{-8}$ and $6.39 \times 10^{-11}$, respectively). The VMR was then used to calculate the dry gas flow (DGF), which is the airflow (L/h) corrected for environmental conditions:

$$DGF = Airflow \times (\frac{100 - VMR}{100})$$

The airflow corrected for environmental conditions was converted to have the airflow in standard condition of temperature and pressure (STP, L/h):

$$STP = \frac{P \times DGF}{T + 273.15} \times \frac{273.15}{1013.25}$$

Finally, $CH_4$ emissions (L/day) were calculated from $CH_4$ concentrations (ppm) and STP (L/h):

$$CH_4\ (L/day)\ =\ CH_4\ (ppm) \times 10^{-6} \times STP \times 24$$

However, the difference between uncorrected and corrected $CH_4$ emissions by environmental parameters was low (~3%), leading us to the conclusion that this correction is not appropriate in our experimental conditions.

## *2.2. Continuous monitoring of rumen dissolved hydrogen concentration: adaptation of a $H_2$-sensor to the rumen environment*

According to the literature review (chapter 1), only two methods allow *in situ* and continuous measurement of dissolved $H_2$ concentrations in the rumen. Hillman et al. (1985) used a Clark-type oxygen electrode placed within the rumen and connected to a mass spectrometer. In the method of Smolenski and Robinson (1988), dissolved $H_2$ is uptaken by a carrier gas passing through a probe immerged into the rumen and connected to a gas chromatograph. These methods have two disadvantages: i) they require important equipment (mass spectrometer) and large-size probes which may disturb the ruminal environment; ii) the response time is quite long (90% response in 2 min) whereas the turnover time of $H_2$ in the rumen is much quicker (0.08 sec). Consequently, we chose to adapt a $H_2$-sensor commonly used in marine research for *in situ* and continuous measurement of dissolved $H_2$ concentration in the rumen.

### 2.2.1. Description of the system and measuring principle

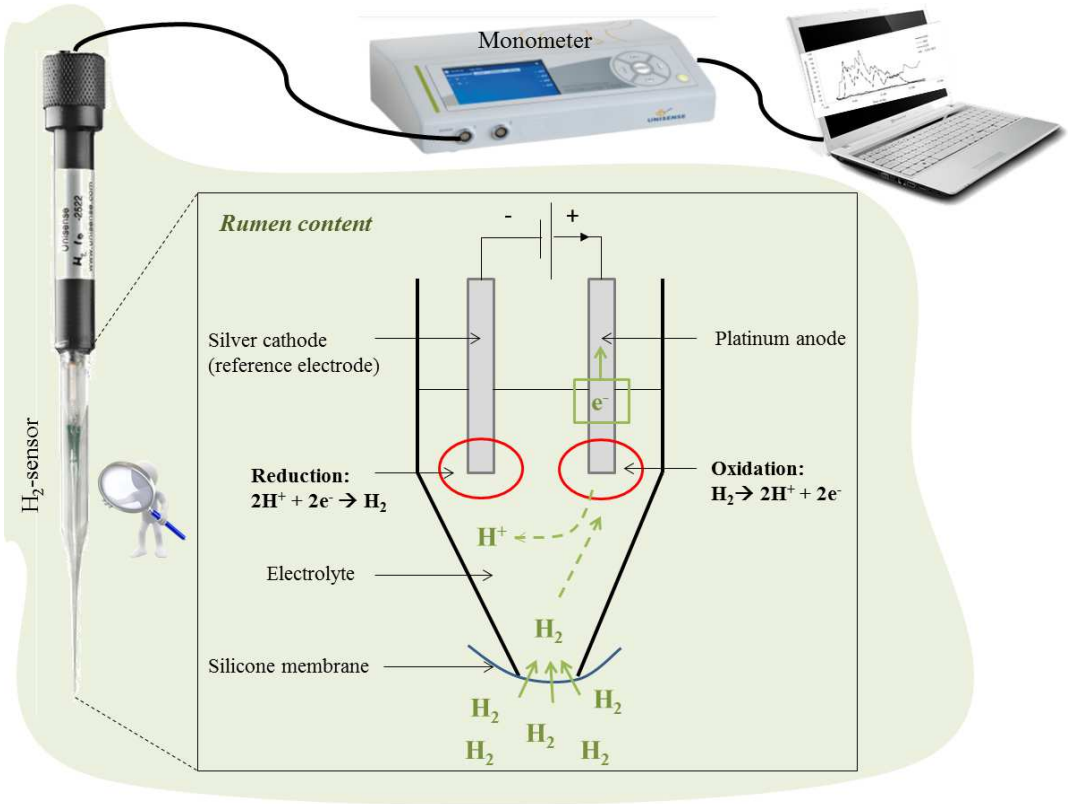


**Figure 17** Description of the system for *in situ* and continuous monitoring of dissolved $H_2$ concentration in the rumen

For *in situ* and continuous measurement of dissolved $H_2$ concentration in the rumen, the system included 3 components (**Figure 17**):

1/ **The $H_2$-sensor** ($H_2$-500, Unisense, Denmark) was 17 cm long and diameters were 22 mm at the top and 0.6 mm at the tip. The limit of quantification of $H_2$ concentration was 0.3 µ*M* and the sensor gave a 90% response in 3-15 sec. The glass-made tip hosted a Clark-type electrode made of a silver cathode (reference electrode) and a platinum anode, which both bathed into a conductive solution (or electrolyte). The tip was closed by a silicone membrane allowing ruminal dissolved $H_2$ to diffuse into the sensor.

2/ **The current amplifier or monometer** (Microsensor Monometer Version 1.0, Unisense, Denmark) generated an electric current flowing in the $H_2$-sensor in a closed-circuit system, from the cathode to the anode, and from the anode to the cathode through the electrolyte. The electrical voltage (800 mV), dependent on the composition of the gas to analyze, was set according to manufacturer instructions.

3/ **The computer set with the Sensor Trace Basic software** (Version 3.1.3., Unisense, Denmark) calculated and recorded dissolved $H_2$ concentrations every second.

Concentration of dissolved $H_2$ was measured in a two-step process:

1/ Dissolved $H_2$ in rumen content diffused into the sensor through the silicone membrane until reaching an equilibrium concentration.

2/ Dissolved $H_2$ was oxidized at the anode. Electrons flowed from the anode to the cathode (opposite direction of the electric current), generating a low-intensity electric signal measured by the monometer. Protons remained in the electrolyte until their reduction with electrons coming out of the cathode.

Then, higher was $H_2$ concentration in the rumen and in the sensor electrolyte, higher was the electric signal generated during electrons flow. In other words, the electric signal measured by the monometer was positively correlated with dissolved $H_2$ concentration.

### 2.2.1. System setup and functioning

According to manufacturer instructions, a pre-polarization period was applied before using the sensor, during which it was simply connected to the monometer set to its electrical voltage (800 mV). This process was essential to let the sensor retrieving a stable and weak baseline, *via* elimination of $H_2$ which could have accumulated in the electrolyte during

storage. Then, longer was the period of non-activity of the sensor, longer was the time required for pre-polarization (from 10 minutes to 8 hours).

After pre-polarization, the sensor was calibrated with a defined gas mixture of $H_2$ and $H_2$-free inert bulk carrier gas (80% $H_2$ - 20% $CO_2$). Knowing that $H_2$ solubility is dependent on salinity and temperature (Wiesenburg and Guinasso, 1979), the sensor was placed in a water bath at 39°C in order to reach similar conditions to the rumen. As the sensor linearly detected partial pressure of $H_2$, a two-point calibration curve was created as recommended by Unisense: the sensor was immerged into the water bath without bubbling (0 µ*M* $H_2$) and the electric signal read by the monometer after stabilization was recorded (first calibration point). Then, the defined gas mixture of $H_2$ was allowed to bubble until stabilization and recording of the electric signal (second calibration point). Knowing that the maximum concentration of dissolved $H_2$ in the rumen is 740.9 µ*M* (see literature review for calculation), the dissolved $H_2$ concentration is 740.9×0.8 = 592.7 µ*M* when a 80% $H_2$ gas is bubbling.

After completing these two steps, the sensor was ready for measurement. Before inserting the sensor into the rumen through the cannula, it was protected with a custom-made plastic cap, and ballasted with a 1-kg weight to ensure continuous measurement of dissolved $H_2$ concentration at the bottom of the rumen (Figure 18).

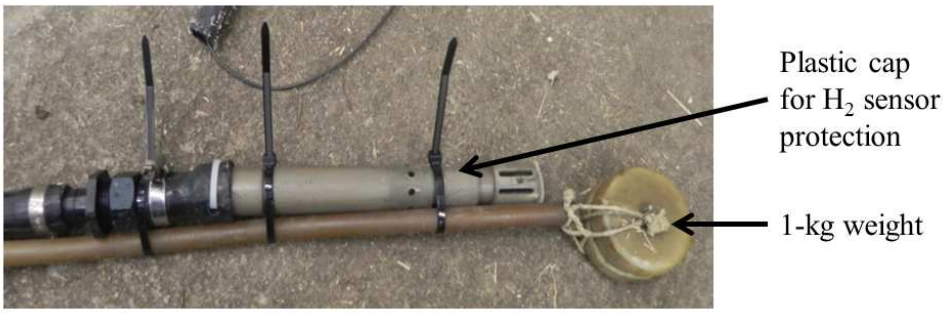


**Figure 18** Protection cap of the $H_2$-sensor

The sensor in its protection was connected to the monometer *via* a 10-m wire extension protected in a plastic tube. After insertion of the sensor into the rumen, the protected wire was attached to the cow with a harness, to make sure the animal cannot move the device. The cannula was closed with a plastic cork to limit rumen liquid and gas leakage (Figure 19).

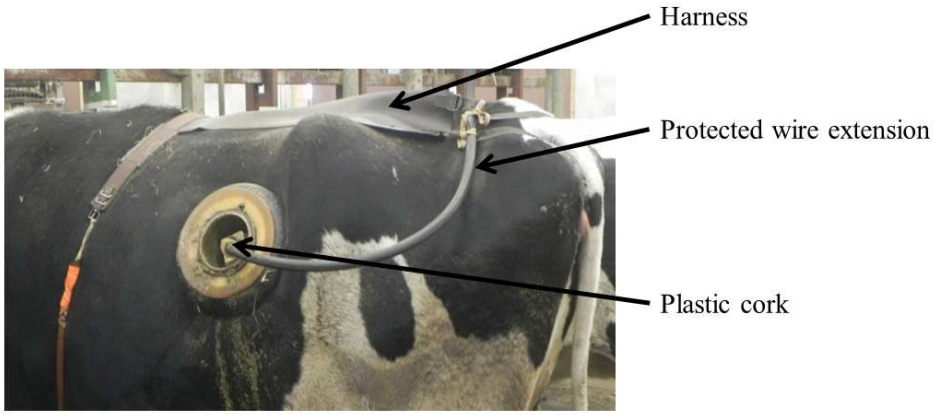


**Figure 19** Hydrogen sensor setup on the animal

# Results

# STEP 1: Additive effect between dietary linseed oil and nitrate as methane emission-reducer in cattle

## Objective

1/ To evaluate the effect of association of feeding strategies acting on $H_2$ production (lipids from linseed, toxic effect towards protozoa) and $H_2$ utilization (nitrate from calcium nitrate, $H_2$-sink through nitrate reduction to nitrite and ammonia) on $CH_4$ emissions, diet digestibility and N balance of non-lactating cows.

2/ To understand the $CH_4$-mitigating effect of these feeding strategies by focusing on rumen $H_2$ pool and fermentation parameters.

## Experimental approach

4 non-lactating cows → 2 × 2 Factorial design →

**CON**: 50% hay + 50% pelleted concentrate
**NIT**: CON + 2.3% nitrate (from calcium nitrate)
**LIN**: CON + 2.6% added lipids (from linseed oil)
**LIN+NIT**: CON + 1.0% added lipids + 2.3% nitrate

4 experimental periods of 5 weeks (wk 1 to 2 = Adaptation; wk 3 to 5 = Measurement)

| WEEK | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Daily intake | ■ | ■ | ■ | ■ | ■ |
| Blood metHb (3 h after morning feeding, once a week) | ■ | ■ | ■ | ■ | |
| Kinetics of rumen dissolved $H_2$ concentrations (during 6 h after morning feeding, one day/cow) | | | ■ | | |
| Total tract digestibility, N balance (6 days)<br>Rumen fermentation (0 and 3 h after morning feeding, twice a week) | | | | ■ | |
| Daily kinetics of $CH_4$ emissions (4 days)<br>Daily kinetics of rumen pH (6 days) | | | | | ■ |

## Main results

| | Diet | | | | | *P*-Value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | NIT | LIN | LIN+NIT | SEM | Nitrate | Linseed | Linseed × nitrate |
| DM intake (kg/day) | 12.4 | 12.3 | 12.3 | 12.2 | 0.59 | 0.22 | 0.35 | 0.86 |
| $CH_4$ emissions (g/kg DM intake) | 25.0 | 19.4 | 20.7 | 17.0 | 0.70 | <0.01 | <0.01 | 0.18 |
| DM digestibility (%) | 63.7 | 64.1 | 64.0 | 63.3 | 0.77 | 0.85 | 0.65 | 0.43 |
| N balance (% of N intake) | 7.4 | 11.8 | 4.0 | 4.8 | 2.25 | 0.20 | 0.03 | 0.35 |
| Rumen protozoa ($log_{10}$/mL, 0 h) | 5.87 | 5.71 | 5.55 | 5.73 | 0.060 | 0.91 | 0.03 | 0.02 |
| Rumen C2/C3 (0 h) | 4.74 | 4.68 | 3.97 | 4.41 | 0.221 | 0.39 | 0.04 | 0.26 |
| Rumen $H_2$ concentrations (µ*M*) | 3.6 | 45.3 | 4.0 | 21.0 | 14.10 | 0.07 | 0.41 | 0.39 |

## Conclusion

Nitrate plus lipids from linseed have an additive $CH_4$-mitigating effect without altering digestibility and N balance. These two dietary strategies have different modes of action on the rumen $H_2$ pool. Further work is necessary to assess the long-term effect of this association on methanogenesis, rumen microbiota and animal performances.

# Additive effect between dietary linseed oil and nitrate as methane emission-reducer in cattle

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**Abstract**

The objective of this study was to test the effect of linseed oil and nitrate fed alone or in combination on methane ($CH_4$) emissions and diet digestibility in cows. The experiment was conducted as a 2 × 2 factorial design using 4 multiparous non-lactating Holstein cows (initial BW 656 ± 31 kg). Each experimental period lasted 5 weeks, with measures performed in the final 3 weeks (wk 3 to wk 5). Diets given on a DM basis were: 1) control (CON, 50% natural grassland hay and 50% concentrate), 2) CON with 4% linseed oil (LIN), 3) CON with 3% calcium nitrate (NIT), 4) CON with 4% linseed oil plus 3% calcium nitrate (LIN+NIT). Diets were offered twice daily and were formulated to deliver similar amounts (DM basis) of CP (12.2%), starch (25.5%) and NDF (39.5%). Feed offer was restricted to 90% of voluntary intake (12.4 kg DMI/d). Total tract digestibility and N balance were determined from total feces and urine collected separately for 6 d during wk 4. Daily $CH_4$ emissions were quantified using open chambers for 4 d during wk 5. Rumen fermentation and microbial parameters were analyzed from samples taken before and 3 h after the morning feed. Rumen concentrations of dissolved hydrogen ($H_2$) were measured continuously up to 6 h post-feeding using a $H_2$ sensor. Compared with CON, linseed oil and nitrate decreased ($P < 0.01$) $CH_4$ emissions (g/kg DMI) by 17 and 22%, respectively, when fed alone and by 32% when combined. The LIN diet reduced $CH_4$ production throughout the day, increased ($P = 0.02$) propionate proportion and decreased ($P = 0.03$) ruminal protozoa concentration compared with CON. The NIT diet strongly reduced $CH_4$ production 3 h post-feeding, with a simultaneous increase in rumen dissolved $H_2$ concentration, suggesting that nitrate does not only act as an electron acceptor. As a combined effect, linseed plus nitrate also increased $H_2$ concentrations in the rumen. Diets had no effect ($P > 0.05$) on total tract digestibility of nutrients, except with linseed oil which tended to reduce ($P < 0.10$) fiber digestibility. Nitrogen balance (% of N intake) was positive for all diets but retention was lesser ($P = 0.03$) with linseed oil. This study demonstrates an additive effect between nitrate and linseed oil for reducing methanogenesis in cows without altering diet digestibility.

**Keywords:** hydrogen, lipid, methane mitigation, nitrate, ruminant

**Introduction**

Enteric methane ($CH_4$) from ruminants is one of the most important greenhouse gas at the farm level (Gerber et al., 2013), and represents an energy loss to the animal (2-12% of GE intake; Johnson and Johnson, 1995). Lipids and nitrate ($NO_3^-$) emerged as persistent and viable dietary options for mitigating $CH_4$ emissions from ruminants (Doreau et al., 2014a). Linseed reduced methanogenesis (-5.6% per 1% added fat; Doreau et al., 2011) but this effect was not always reported (Chung et al., 2011; Veneman et al., 2014). Linseed, rich in polyunsaturated fatty acids (PUFA), may improve animal product quality (Scollan et al., 2001; Chilliard et al., 2009), but fat doses greater than 5% may lower animals' performance (McGinn et al., 2004; Martin et al., 2008). In the diet, $NO_3^-$ repeatably reduced $CH_4$ emissions (-10% per 1% added $NO_3^-$; Lee and Beauchemin, 2014), but its use as a urea substitute still requires investigations into its possible impacts on animal health, digestive parameters and residuals in animal products for human consumption.

In the rumen, $CH_4$ is mainly produced by methanogens using carbon dioxide ($CO_2$) and hydrogen ($H_2$). Both are fermentation end-products, but as $H_2$ is limiting, modulating its concentration could reduce methanogenesis (Hegarty and Gerdes, 1999). Linseed and $NO_3^-$ affect the rumen $H_2$ pool in unique ways. Linseed reduces $H_2$ production mainly through its toxic effect against rumen protozoa, which are major $H_2$ producers (Morgavi et al., 2010). As fat is not fermented in the rumen, substitution of rumen fermentable substrates for lipids may also reduce $H_2$ production. To a lesser degree, PUFA can reduce $H_2$ availability in the rumen by consuming $H_2$ during biohydrogenation (Czerkawski, 1986). Nitrate modifies $H_2$ consumption by reducing the number of methanogens (Van Zijderveld et al., 2010) and by acting as a $H_2$-sink (Lewis, 1951).

As these dietary treatments share different mechanisms of action, we hypothesized that their combination would have an additive effect that leads to lesser net methanogenesis than when they are individually fed. However, as a feeding strategy should reduce $CH_4$ emissions without adverse effect on animals' digestive efficiency, performance and health, our hypothesis was tested in an in vivo experiment with dry cows designed to evaluate the effect of linseed plus nitrate on: 1) $CH_4$ emissions and mechanisms involved in methanogenesis (rumen $H_2$ pool and fermentation); 2) diet digestibility and nitrogen balance.

**Materials and methods**

The experiment was conducted at the animal facilities of the Experimental Unit UERT at the INRA's Theix Research Centre (Saint-Genès-Champanelle, France) from January to June 2013. Procedures involving animals were performed in accordance with French Ministry of Agriculture guidelines for animal research and with the applicable EU guidelines and regulations on experiments with animals. The experiment was approved by the local Auvergne-region ethics committee on animal experimentation, approval number CE50-12.

*Animals, experimental design and diets*

Four multiparous non-lactating Holstein cows fitted with rumen cannulas (initial average BW of 656 ± 31 kg and age of 6.7 ± 1.5 years, mean ± SD) and habituated to handling were housed in individual stalls during the experiment. The cows were randomly assigned to 4 dietary treatments in a 2 × 2 factorial design, using either calcium nitrate or linseed oil at two different doses (0 and 3% for calcium nitrate; 0 and 4% for linseed oil). Each experimental period lasted 5 weeks, with measures performed in the final 3 weeks (wk 3 to wk 5). The diets, given on a DM basis, were: 1) control (CON), 2) CON with 4% linseed oil (LIN), 3) CON with 3% calcium nitrate (NIT), 4) CON with 4% linseed oil and 3% calcium nitrate (LIN+NIT). The doses of linseed oil (Vandeputte Savonnerie et Huilerie, Mouscron, Belgium) and calcium nitrate (75% $NO_3^-$ in DM; Phytosem, Pont-du-Château, France) were calculated to achieve a theoretical $CH_4$ reduction of 20% when distributed alone (Martin et al., 2008; Van Zijderveld et al., 2011; Hulshof et al., 2012).

Ingredients and chemical composition of the experimental diets are reported in Table 1. The CON diet consisted of 50% natural grass hay (harvested in semi-mountainous and permanent grassland areas) and 50% concentrate (DM basis). Diets were formulated at the beginning of the experiment to meet at least the ME requirements for maintenance of non-lactating cows (INRA, 2010) and to get sufficient and similar levels of NDF (to avoid any risk of acidosis; Krause and Oetzel, 2006), starch (to favor protozoa development; Jouany, 1989), and CP. Diet levels of fermentable N were kept moderate in order to assess the effect of nitrate on N output. Diets were adjusted to have the same N and Ca concentrations by including urea and calcium carbonate in the non-NIT diets (i.e. CON and LIN). Calcium carbonate was used as it has low solubility in the rumen and thus avoids the formation of calcium salts with lipids (Keyser et al., 1985). A commercial mineral-vitamin premix was added in equal amounts to

all diets. Forage was distributed without further processing. All other ingredients including linseed oil or nitrate or both were pelleted in concentrates (InVivo NSA, Chierry, France).

**Table 1** Ingredients and chemical composition of the experimental diets

| | Diet[1] | | | |
|---|---|---|---|---|
| Item | CON | NIT | LIN | LIN+NIT |
| Ingredient, % of DM | | | | |
| Hay | 50.00 | 50.00 | 50.00 | 50.00 |
| Pelleted concentrate | | | | |
| Wheat | 25.23 | 25.23 | 25.23 | 25.23 |
| Maize | 15.00 | 15.00 | 15.00 | 15.00 |
| Calcium nitrate[2] | 0 | 3 | 0 | 3 |
| Linseed oil | 0 | 0 | 4 | 4 |
| Calcium carbonate | 1.7 | 0 | 1.7 | 0 |
| Urea | 1.22 | 0 | 1.22 | 0 |
| Dehydrated beet pulp | 4.08 | 4 | 0.08 | 0 |
| Molasses beet | 1 | 1 | 1 | 1 |
| Binder | 1 | 1 | 1 | 1 |
| Mineral-vitamin mix | 0.75 | 0.75 | 0.75 | 0.75 |
| Aroma | 0.02 | 0.02 | 0.02 | 0.02 |
| Chemical composition | | | | |
| OM, % of DM | 91.3 | 91.5 | 91.8 | 91.8 |
| CP, % of DM | 12.7 | 12.2 | 12.1 | 11.7 |
| NDF, % of DM | 40.1 | 40.2 | 38.8 | 38.7 |
| ADF, % of DM | 23.3 | 23.1 | 22.2 | 22.2 |
| Starch, % of DM | 25.4 | 25.7 | 25.7 | 25.3 |
| Ether extract, % of DM | 2.08 | 1.90 | 4.66 | 3.12 |
| Total fatty acids, % of DM | 1.61 | 1.24 | 3.53 | 2.05 |
| GE, MJ/kg of DM | 17.4 | 16.6 | 18.3 | 17.7 |
| Fatty acid, % of total fatty acids | | | | |
| C16:0 | 18.56 | 24.55 | 14.18 | 20.38 |
| C18:0 | 1.98 | 2.58 | 4.92 | 6.56 |
| C18:1 n-9 | 19.53 | 22.90 | 23.13 | 28.60 |
| C18:2 n-6 | 47.50 | 29.33 | 24.89 | 21.22 |
| C18:3 n-3 | 8.01 | 7.72 | 29.37 | 17.63 |

[1] CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.
[2] $5Ca(NO_3)_2.NH_4NO_3.10H_2O$; 75% $NO_3^-$ in DM.

*Feeding and management*

Two weeks before starting the experiment, cows were fed CON ad libitum. Then, throughout the experiment, offered feed was restricted to 90% of individual voluntary feed intakes (1.8 times ME requirements for maintenance) to ensure complete consumption. The LIN, NIT and

LIN+NIT concentrates were progressively supplied by replacing the CON concentrate. The LIN concentrate was distributed at maximal dose after a 5-d transition period. The NIT and LIN+NIT concentrates were distributed at their maximal dose after a 10-d transition period.
Throughout the experiment, feed was offered twice daily (66% at 0800 h and 34% at 1600 h for hay; 60% between 0800 and 0930 h in 3 equal portions and 40% between 1600 and 1630 h in 2 equal portions for concentrates). Distribution of concentrates was fractionated to reduce the risk of methemoglobinemia (metHb; Morris et al., 1958). Forage-to-concentrate ratio (50:50) was kept as close as possible to the target ratio by adjusting the amounts of hay and concentrates offered daily. Cows had free access to water throughout the experiment.

*Measurements and analyses*

*Intake.* Feed intake was weighed and recorded daily throughout the experiment to estimate DMI. There were no refusals during the experiment. Samples of each feed (200 g of hay and concentrates) were taken on 2 days in wk 4 and wk 5 of each period. One sub-sample was used to determine DM content (103°C for 24 h) and another sub-sample was stored at 4°C before being pooled at the end of the experiment. These pooled samples were ground down using an Ultra Centrifugal Mill (0.75 mm sieve; Retsch GmbH, Haan, Germany) and analyzed for chemical composition.
Organic matter was determined by ashing at 550°C for 6 h (method 942.05; AOAC, 2005). Total N was analyzed by combustion according to the Dumas method (method 968.06; AOAC, 2005), and CP content was calculated as N × 6.25. Fiber (NDF and ADF) was determined by sequential procedures (Van Soest et al., 1991) after pretreatment with amylase, and expressed exclusive of residual ash. Starch was analyzed using an enzymatic method (Faisant et al., 1995). The GE was analyzed by isoperibolic calorimetry (C200 model, IKA, Staufen, Germany). Ether extract (EE) was determined after acid hydrolysis (method 954.02; AOAC, 2005), and fatty acid (FA) composition was determined by gas chromatography of methyl esters (method 969.33; AOAC, 2005).

*Cow liveweights and methemoglobinemia.* Cows were weighed at the beginning of the experiment and at the end of each experimental period. Levels of blood metHb were measured on all cows 3 h after morning feeding (1100 h) on the day before the start of the experiment (control blood) and then at d 3 and 5 (1% calcium nitrate in the diet), d 10 (2% calcium nitrate in the diet) and d 12, 17, 19 and 22 (3% calcium nitrate in the diet) of each experimental

period for cows fed NIT and LIN+NIT. Blood from cows fed CON and LIN was not analyzed as we assumed that there was no risk of metHb. Blood (10 mL) was sampled from the jugular vein into K2-EDTA collection tubes (Venosafe, Terumo, Guyancourt, France) and packed on ice for metHb content to be determined by spectrophotometry (UV-160, Shimadzu, Marne-La-Vallée, France) within 1 h at the nearest hospital (CHU Gabriel Montpied, Clermont-Ferrand, France; method of Kaplan, 1965). A metHb threshold value was set at 30% hemoglobin (Hb). Any animal meeting this cut-off would be removed from the experiment and treated with 1% methylene blue (The United States Pharmacopeial Convention, 2008).

*Diet digestibility and nitrogen balance.* Total tract digestibility and N balance were determined from total and separate collection of feces and urine for 6 days during wk 4 of each experimental period. To separate urine from feces, cows were fitted with flexible pipes (Doreau et al., 2014b) connected to a 30-L flask containing 500 mL of 3 M sulfuric acid to achieve a urine pH < 3 and thus avoid N volatilization. Feces and urine were removed once daily.

Each morning, after weighing and mixing of feces, a 1% fresh aliquot was used for DM determination (103°C for 24 h) and a 0.5% fresh aliquot was pooled across days for each animal and frozen (-20°C). At the end of the experiment, pooled samples were thawed, dried (60°C for 72 h) and ground (1-mm screen) to determine OM, N, NDF and ADF content as previously described.

Each morning, after weighing urine, a 0.5% fresh aliquot was pooled across days for each animal and frozen (-20°C). At the end of the experiment, the N content of thawed urine was determined by the Kjeldahl method (method 2001.11; AOAC, 2005) as it was impossible to apply the Dumas method on fresh urine.

*Rumen fermentation parameters.* Total rumen contents were sampled (~200 g) from the ventral sac through the cannula before (0745 h) and 3 h after (1100 h) the morning feed on 2 non-consecutive days (d 3 and 5) in wk 4 of each experimental period. The samples were strained through a polyester monofilament fabric (250 μm pore size) and filtrate was subsampled for subsequent analyses. For VFA analysis, 0.8 mL of filtrate was mixed with 0.5 mL of a 0.5 M HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid. For ammonia-nitrogen ($NH_3$-N) analysis, 1 mL of filtrate was mixed with 0.1 mL of 5% orthophosphoric acid. For lactate and nitrate-nitrite concentrations analysis, 3 mL

and 20 mL of filtrate, respectively, were collected without preservative (Sar et al., 2004). All these samples were stored at -20°C until analysis. For protozoa counts, 2 mL of filtrate was mixed with 2 mL of methyl green-formalin solution and stored away from direct light until counting.

Concentrations of VFA and $NH_3$-N were analyzed by gas chromatography with a flame ionization detector and by colorimetry, respectively (Morgavi et al., 2008). Lactate concentrations were determined by colorimetry (D/L-lactic acid, BioSentec, Auzeville-Tolosane, France). Nitrate and nitrite concentrations were analyzed by colorimetry (method EPA 353.2; SmartChem 200, Unity Scientific, Brookfield, USA; Laboratoire Vétérinaire et Biologique, Lempdes, France). Protozoa were counted by microscopy and categorized as either small (< 100 µm) or large (> 100 µm) entodiniomorphs, or as holotrichs (*Dasytricha* or *Isotricha*) (Williams and Coleman, 1992). Data for protozoa were $\log_{10}$-transformed before statistical analysis.

*Monitoring pH and dissolved $H_2$ concentration in the rumen.* Rumen pH was monitored continuously over wk 5 using commercial boluses (eBolus, eCow, Exeter, UK). One day before measurement, the boluses were calibrated using buffer solutions (pH 4 and 7; HM Digital, Culver City, CA). One bolus per cow was immersed in the ventral sac of the rumen. Data were then recorded every 15 min during 6 full days, after which the boluses were removed. At the end of each experimental period, data were uploaded by telemetry to a digital tablet before being transferred to a computer.

The dynamics of dissolved $H_2$ concentrations in the rumen were successively measured on each cow in wk 3 (one day per cow) with a $H_2$ sensor (H2-500, Unisense, Denmark). The electrode was connected to a microsensor monometer via a 10-m wire extension (Unisense, Denmark), and the monometer was connected to a portable computer running Sensor Trace Basic software (Version 3.1.3; Unisense, Denmark). The sensor was polarized (800 mV) once in wk 3 (8 h before the start of measurement) and calibrated daily by immersion in a water bath at 39°C bubbling with a 80% $H_2$/20% $CO_2$ gas mixture. The sensor and wire extension were protected using a custom-made plastic cap and tube (Figure 1). The system was ballasted with a 1-kg weight and introduced into the cow's ventral sac through the cannula at 30 min before the morning feed (i.e. 0730 h). The setup was fitted taking care to avoid gas and liquid leakage from the rumen. Dissolved $H_2$ concentration readings were recorded every second for

6 h after the morning feed. For an easier use of the sensor, it was essential to remove it when the rumen was not full i.e. before the afternoon feeding.

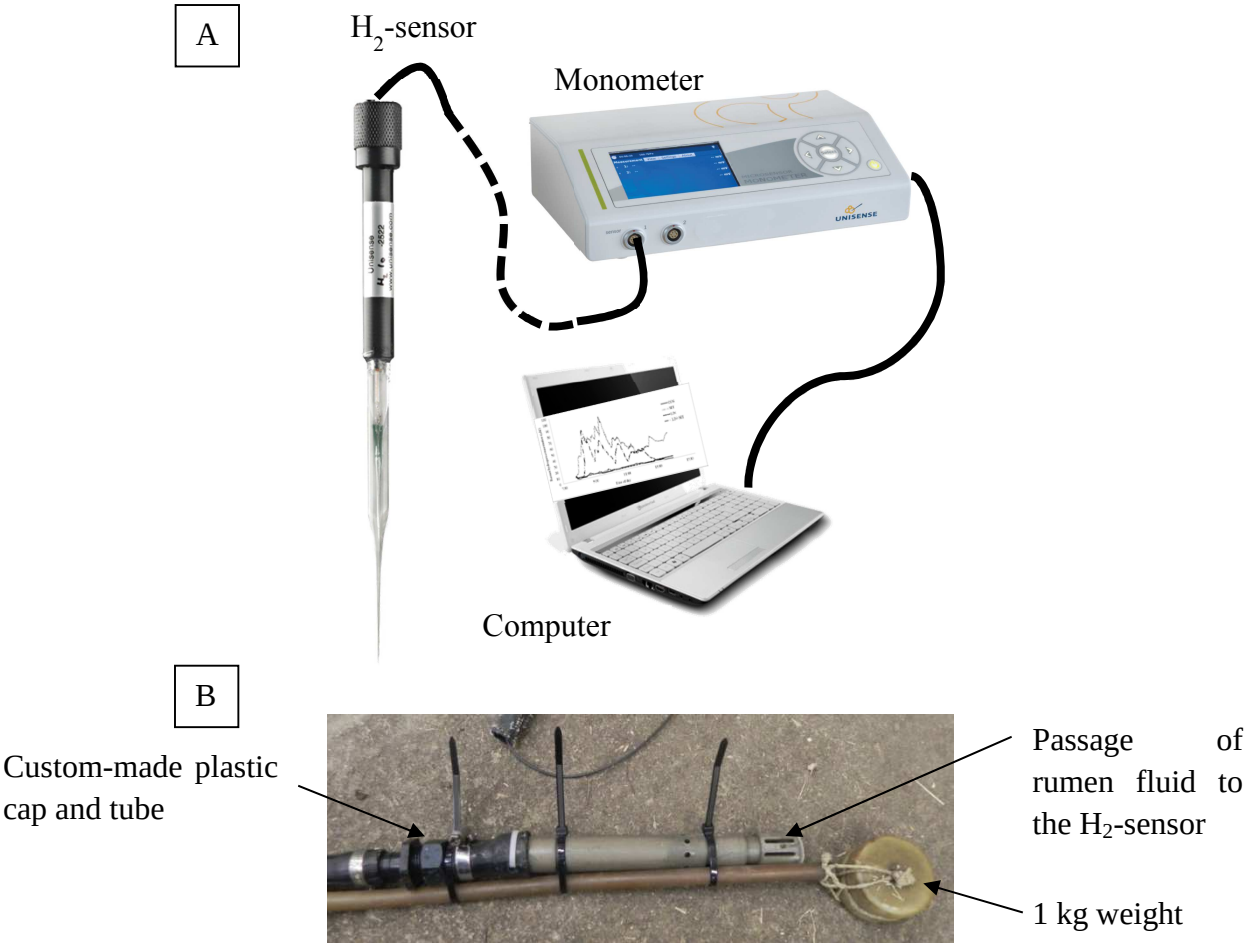



**Figure 1.** Use of $H_2$-sensor (Unisense, Denmark): A. Overall setup with sensor, monometer and computer; B. Protection cap of the sensor.

*Methane and carbon dioxide emissions.* In wk 5, animals were placed in open chambers (1 animal/chamber) for 4 consecutive days. Individual total $CH_4$ and $CO_2$ emissions were measured continuously from d 1 (0730 h) to d 5 (0730 h).

Each chamber was 2.2 m high, 3.6 m long and 2.1 m wide, giving a volume of 16.6 $m^3$. The chambers were made of steel with clear polycarbonate walls allowing sight contact between animals and with the farm personnel. Chambers had front and rear doors, with the front doors used for animal feeding and the rear doors used to enter the animals and to remove feces and urine collected in a wheeled recovery box. Front and rear doors were never simultaneously opened in order to avoid an air stream into the chamber. The feces and urine recovery boxes were removed each morning and immediately replaced with new ones in order to minimize

chamber opening time (5 min per chamber on average). When rear doors were closed, front doors were opened (5 min per chamber on average) for morning (1 portion of hay at 0800h, 3 portions of concentrates at 0800, 0830 and 0930h) and afternoon (1 portion of hay at 1600h, 2 portions of concentrates at 1600 and 1630h) feeding.

The chambers operated at a slight negative pressure, with an air flow oscillating between 700 and 800 $m^3/h$ (approximately 45 air changes per h). Airflow entered the chamber through an aperture at the bottom of the rear door (0.42 $m^2$) and exited through an exhaust duct situated at the top of the chamber, over the head of the animal. Airflow in the exhaust duct of each chamber was continuously measured (CP300, KIMO, Montpon-Ménestérol, France) and recorded with one datapoint every 5 min (KT-210-AO, KIMO, Montpon-Ménestérol, France). Concentration of gases in the barn and in the 4 chambers was alternatively analyzed at a 0.1 Hz sample frequency for 5 min every 25 min using an infrared detector (Ultramat 6, Siemens, Karlsruhe, Germany) and recorded (Nanodac Invensys, Eurotherm Automation SAS, Dardilly, France). The detector was manually calibrated the day before each measurement period using pure $N_2$ and a mixture of $CH_4$ (650 ppm) and $CO_2$ (700 ppm) in $N_2$. Missing data between 2 measurement intervals were recovered by linear regression. Chamber doors were never opened during gas analysis, so no data was deleted. Real-time gas emissions in a chamber were calculated by the difference between chamber and ambient gas concentrations multiplied by the airflow corrected for temperature, relative humidity and pressure according to the Wexler equation (Pinares-Patiño et al., 2012).

*Statistical analyses*

Except for metHb, data were analyzed using the MIXED procedure of SAS (Version 9.2; SAS Institute, 2009). Gaseous emissions ($CH_4$ and $CO_2$) and rumen fermentation parameters measured during several days (n = 4 and 2 days, respectively) were averaged per period before being included in the statistical analyses. The model included the random effect of cow (n = 4) and fixed effects of period (n = 4), nitrate (CON and LIN versus NIT and LIN+NIT), linseed (CON and NIT versus LIN and LIN+NIT) and the interaction nitrate × linseed. Rumen fermentation data obtained before and after feeding (VFA, $NH_3$-N, lactate, protozoa, nitrate and nitrite concentrations) were analyzed using the same model and for the 2 sampling hours separately. Continuous measurements of ruminal pH, dissolved $H_2$ concentrations and $CH_4$ emissions were analyzed by repeated time. Several covariance structures were compared, and compound symmetry (CS) was selected as it resulted in the lowest values for the Akaike's

information criteria. The model included the fixed effects of period, hour, nitrate, linseed, nitrate × linseed and the interactions between hour and dietary treatments (linseed × hour, nitrate × hour, linseed × nitrate × hour). Differences among treatments were tested using the PDIFF option. Data were considered significant at $P < 0.05$. Least squares means are reported throughout.

## Results

*Liveweight and blood methemoglobin*

Animals gained on average 26.5 kg per experimental period, with a final BW at the end of the trial of 762 ± 47 kg. For diets containing nitrate (NIT and LIN+NIT), blood metHb gradually increased the first 12 d of adaptation period, but no animal exceeded 26.3% metHb (Figure 2).

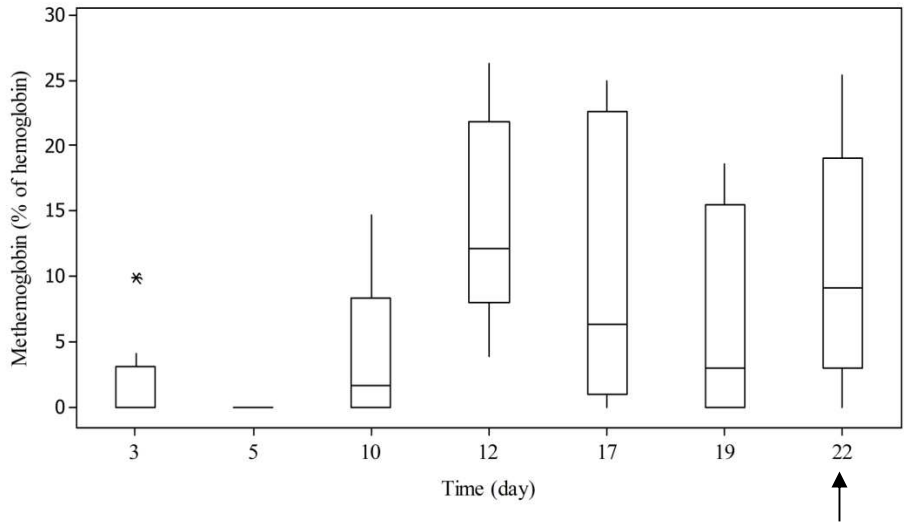



**Figure 2** Boxplot of blood metHb levels of non-lactating cows fed diets containing 3% calcium nitrate with or without 4% linseed oil (n = 8). The box represents the quartiles with the median at the center and the vertical lines represent the maximum and minimum value within 1.5 interquartile range of the higher and lower quartile, respectively. Values greater than 1.5 interquartile range are considered as outliers and are identified with a star. Blood was analyzed during the 3-wk adaptation period, the arrow indicates the start of the measurement period.

*Methane and carbon dioxide emissions*

Dry matter intake of cows while in chambers was the same as outside, showing the absence of stress of animals, and that $CH_4$ determination in our experimental conditions accurately reflected emissions throughout the trial. Methane production was different among diets

irrespective of the unit of expression (Table 2; $P < 0.01$). Compared with CON, $CH_4$ (g/d) was 18, 23 and 33% lesser for LIN, NIT and LIN+NIT, respectively. An additive $CH_4$-mitigating effect between linseed and nitrate (linseed × nitrate, $P > 0.05$) was observed when $CH_4$ was expressed as a function of DMI, digested DM, digested OM or as a percentage of GE intake. When expressed per kg of digested NDF, $CH_4$ emissions from cows fed nitrate-containing diets were lesser than emissions from cows fed other diets ($P = 0.01$). With LIN+NIT, $CH_4$ emissions were close to those of animals fed NIT showing the absence of additive effect between nitrate and linseed.

Diets affected the daily pattern of $CH_4$ emissions in different ways (Figure 3). For CON, 2 peaks of $CH_4$ production were observed at around 2 h after feeding, with the largest peak after the morning feeding that represented 66% of the total daily ration. The $CH_4$ emissions pattern of LIN was similar to CON but emissions of LIN were consistently lesser throughout the day. In contrast to CON, with NIT and LIN+NIT, the peaks were not observed, and $CH_4$ emissions increased at 3 h post-feeding. Contrary to $CH_4$, $CO_2$ emissions (g/d or g/kg DMI) were not affected by dietary treatments.

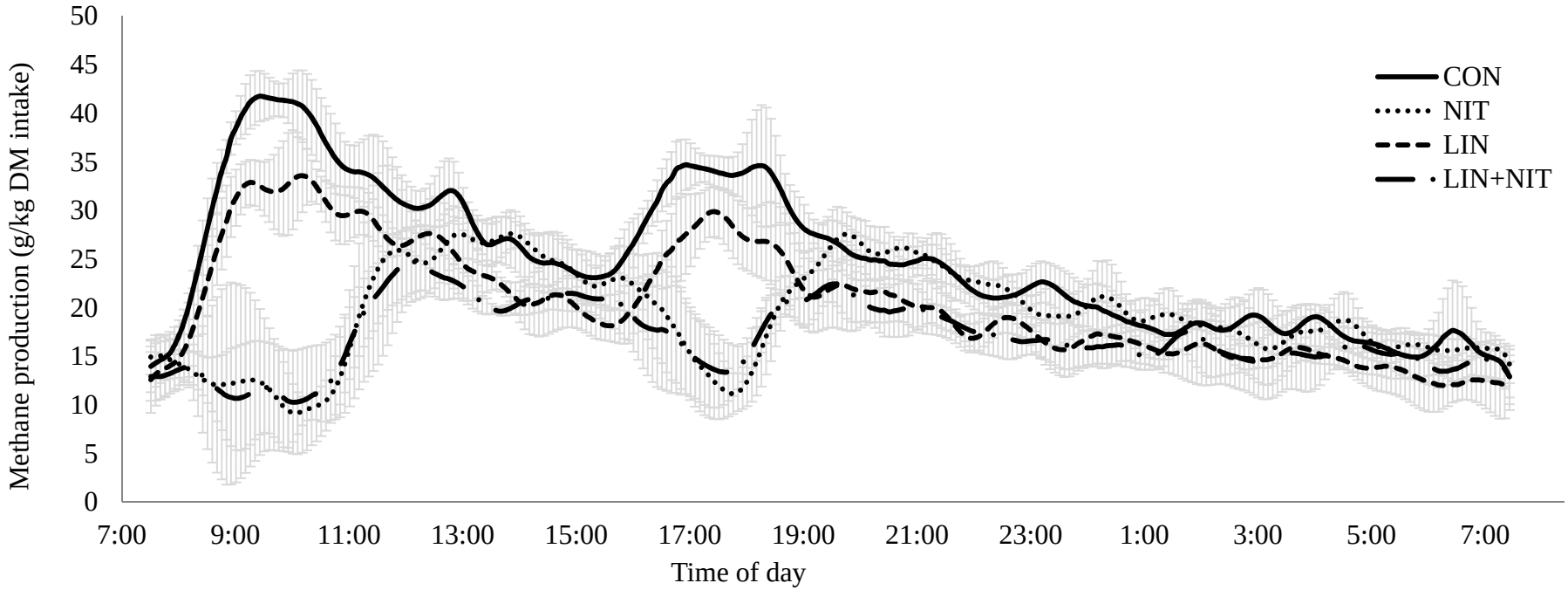
**Table 2** Methane and carbon dioxide emissions of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

| | Diet[2] | | | | | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|---|
| Item[1] | CON | NIT | LIN | LIN+NIT | SEM | Nitrate | Linseed | Linseed × nitrate |
| DM intake, kg/d | 12.4 | 12.3 | 12.3 | 12.2 | 0.59 | 0.22 | 0.35 | 0.86 |
| Methane emissions | | | | | | | | |
| g $CH_4$/d | 308.6 | 238.1 | 252.7 | 206.8 | 9.61 | <0.01 | <0.01 | 0.08 |
| g $CH_4$/kg DM intake | 25.0 | 19.4 | 20.7 | 17.0 | 0.70 | <0.01 | <0.01 | 0.18 |
| g $CH_4$/kg digested DM | 39.3 | 30.3 | 32.4 | 27.0 | 1.18 | <0.01 | <0.01 | 0.14 |
| g $CH_4$/kg digested OM | 36.8 | 28.3 | 30.3 | 25.1 | 1.06 | <0.01 | <0.01 | 0.12 |
| g $CH_4$/kg digested NDF | 55.9 | 43.1 | 47.1 | 43.1 | 2.42 | 0.01 | 0.06 | 0.07 |
| % of GE intake | 7.2 | 5.8 | 5.6 | 4.8 | 0.20 | <0.01 | <0.01 | 0.24 |
| Carbon dioxide emissions | | | | | | | | |
| g $CO_2$/d | 9191 | 9323 | 8988 | 8789 | 562.1 | 0.84 | 0.06 | 0.35 |
| g $CO_2$/kg DM intake | 745 | 757 | 732 | 721 | 28.1 | 0.98 | 0.19 | 0.49 |

[1] Data were collected during 4 consecutive days in wk 5.

[2] CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

[3] Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.



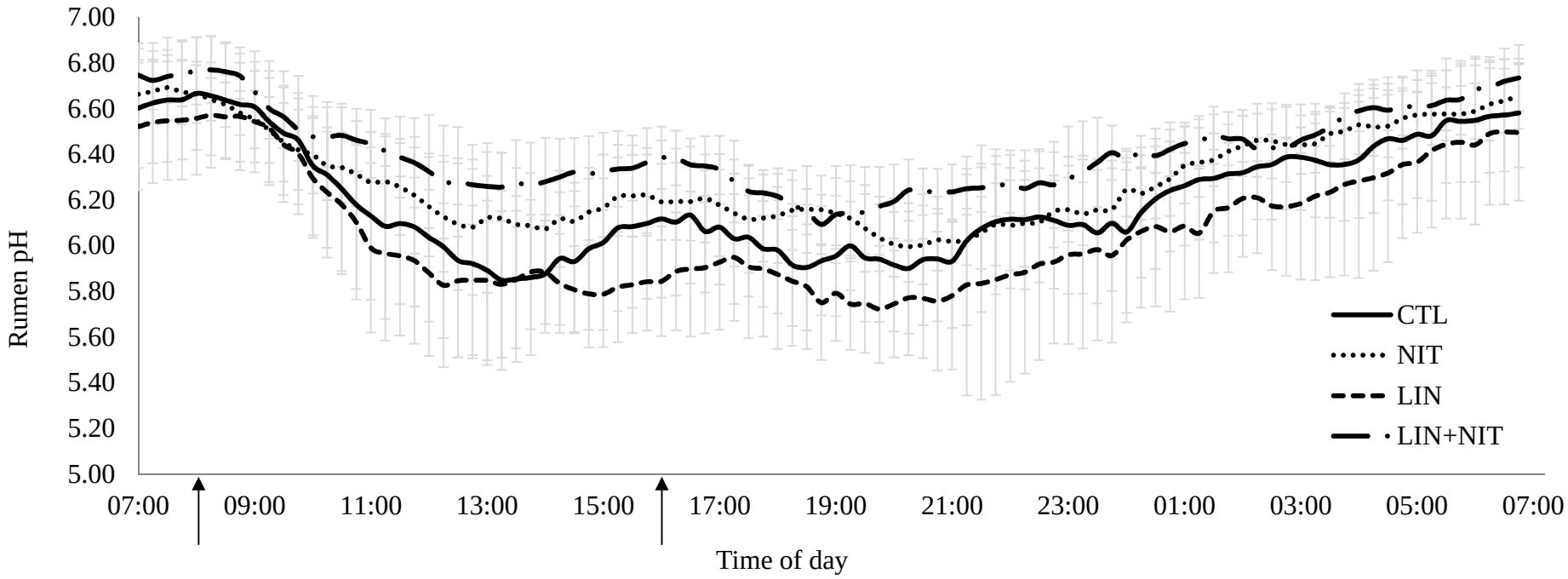

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nitrate | ns | *** | *** | *** | * | * | ns | ns | † | *** | *** | ** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Linseed | ns | *** | *** | * | * | *** | ** | † | *** | *** | *** | ** | ns | * | ** | * | † | ns | ns | * | † | ns | ns | ns |
| Linseed × nitrate | ns | *** | *** | *** | *** | *** | *** | ns | ** | *** | *** | *** | * | * | * | ** | * | ns | ns | * | * | ns | ns | ns |

**Figure 3** Daily methane production pattern of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4). Errors bars indicate SD. Treatments consisted of control diet (CON), CON containing 3% calcium nitrate (NIT), CON containing 4% linseed oil (LIN) and CON containing 4% linseed oil and 3% calcium nitrate (LIN+NIT). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison (†$P$<0.10; *$P$<0.05; **$P$<0.01; ***$P$<0.001) between treatments: linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); linseed × nitrate = interaction between main effects of linseed and nitrate.

*Rumen fermentation parameters*

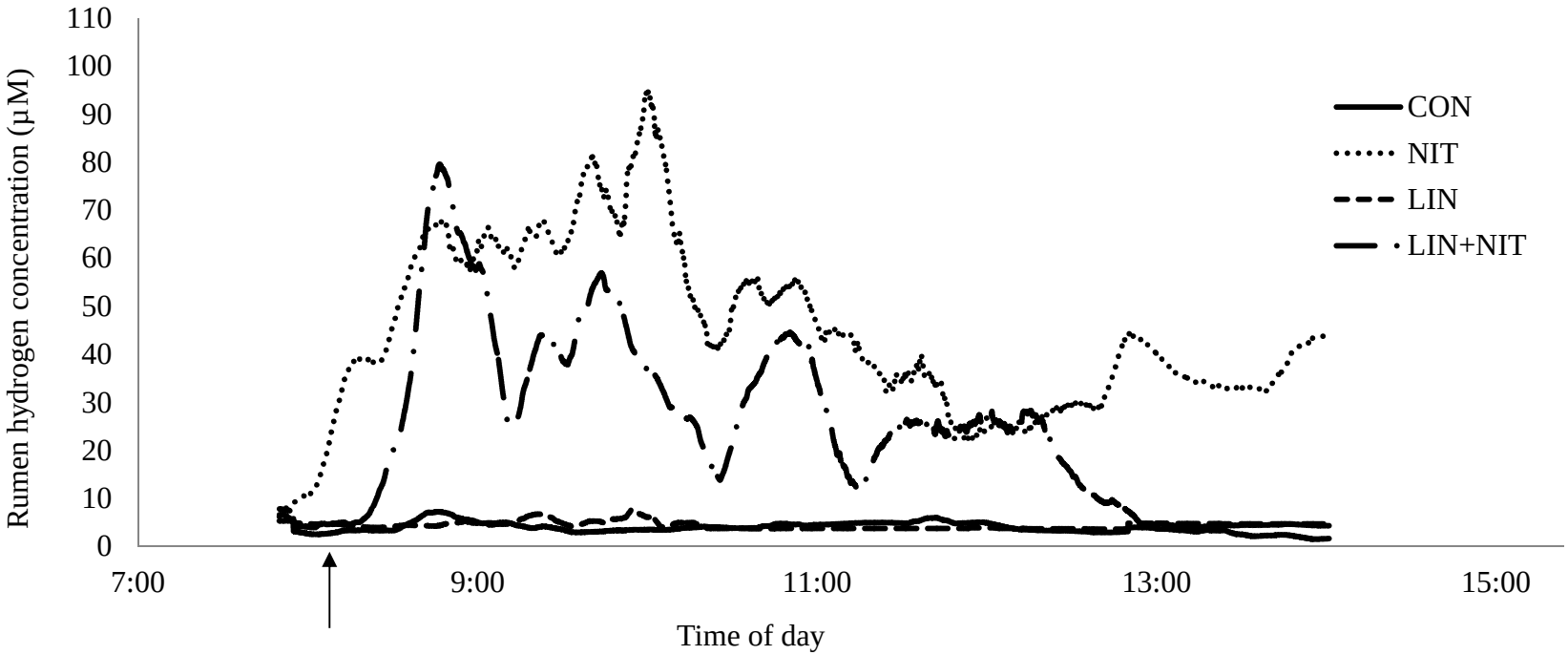
Mean rumen pH was greater for NIT and LIN+NIT compared with CON and LIN (Table 3; +0.23 units on average; $P$ = 0.03). Diet LIN+NIT showed significantly greater pH values compared with CON during daytime, starting 3 h after the morning feeding (Figure 4). Mean dissolved $H_2$ concentrations in the rumen tended ($P$ = 0.07) to be greater for diets including nitrate compared with other diets (+89%). The $H_2$ concentration was constantly low up to 6 h post-feeding for CON and LIN (3.8 µ*M*; Figure 5) but showed a significant jump as early as 1 h post-feeding nitrate (NIT and LIN+NIT). Hydrogen concentrations started to decrease 2 h post-feeding for LIN+NIT and at 3 h post-feeding for NIT. Compared with CON, $H_2$ concentrations were on average 5.9 and 12.6 times greater for LIN+NIT and NIT treatments, respectively.

Concentrations of total VFA were similar among diets before and after feeding. Linseed-containing diets increased propionate proportions before and after feeding ($P$ = 0.02), leading to lesser acetate: propionate and (acetate + butyrate): propionate ratios compared with other diets. Nitrate-containing diets modified VFA profiles after feeding only ($P$ = 0.01), with greater acetate and lesser propionate proportions, inducing greater acetate: propionate and (acetate + butyrate): propionate ratios compared with other diets. At least, nitrate-containing diets increased $NH_3$-N (+20%; $P$ = 0.04) concentrations before feeding. Nitrate concentrations in the rumen were lesser than the limit of quantification (13.3 mg/L or 0.22 m*M*).




| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nitrate | ns | ns | ns | ns | ns | * | † | ns | ns | ns | ns | † | ns | ns | ns | ns | † | ns | ns | ns | ns | ns | ns | ns |
| Linseed | ns | ns | ns | ns | ns | ns | ns | * | * | ns | ns | ns | ns | ns | * | ns | ns | ns | ns | † | ns | ns | ns | ns |
| Linseed × nitrate | ns | ns | ns | ** | ** | *** | *** | ** | * | * | * | † | * | ** | ns | † | ** | † | ns | ns | * | ns | ns | ns |

**Figure 4** Daily pattern of rumen pH of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n= 4). Errors bars indicate SD. Treatments consisted in control diet (CON), CON containing 3% calcium nitrate (NIT), CON containing 4% linseed oil (LIN) and CON containing 4% linseed oil and 3% calcium nitrate (LIN+NIT). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison (†$P$<0.10; *$P$<0.05; **$P$<0.01; ***$P$<0.001) between treatments: linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); linseed × nitrate = interaction between main effects of linseed and nitrate.




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Nitrate | ns | ** | *** | † | ns | ns | ns |
| Linseed | ns | ns | ns | ns | ns | ns | ns |
| Linseed × nitrate | ns | * | ns | ns | ns | ns | ns |

**Figure 5** Rumen dissolved hydrogen concentrations up to 6 h after feeding non-lactating cows with diets containing linseed oil and calcium nitrate alone or in association (n = 4). Treatments consisted in control diet (CON), CON containing 3% calcium nitrate (NIT), CON containing 4% linseed oil (LIN) and CON containing 4% linseed oil and 3% calcium nitrate (LIN+NIT). The arrow indicates time of morning feeding. Symbols indicate hourly statistical comparison (†$P$<0.10; *$P$<0.05; **$P$<0.01; ***$P$<0.001) between treatments: linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); linseed × nitrate = interaction between main effects of linseed and nitrate.

**Table 3** Rumen fermentation characteristics of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

| Item[1] | Time after feeding (h) | Diet[2] | | | | SEM | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CON | NIT | LIN | LIN+NIT | | Nitrate | Linseed | Linseed × nitrate |
| Total VFA, m*M* | 0 | 73.8 | 72.7 | 69.4 | 71.4 | 6.42 | 0.93 | 0.56 | 0.75 |
| | 3 | 111.9 | 102.6 | 102.6 | 107.7 | 6.52 | 0.74 | 0.74 | 0.28 |
| VFA composition, mol/100 mol | | | | | | | | | |
| Acetate (A) | 0 | 70.9 | 69.5 | 69.5 | 69.6 | 1.00 | 0.53 | 0.53 | 0.43 |
| | 3 | 70.2 | 73.4 | 67.0 | 73.1 | 1.07 | 0.01 | 0.15 | 0.23 |
| Propionate (P) | 0 | 15.0 | 15.0 | 17.6 | 16.0 | 0.59 | 0.20 | 0.02 | 0.23 |
| | 3 | 15.8 | 14.8 | 19.4 | 15.4 | 0.95 | 0.01 | 0.02 | 0.06 |
| Butyrate (B) | 0 | 10.3 | 11.4 | 9.0 | 10.4 | 0.71 | 0.08 | 0.11 | 0.81 |
| | 3 | 10.4 | 8.7 | 10.1 | 8.4 | 1.20 | 0.19 | 0.82 | 0.98 |
| Minor VFA[4] | 0 | 3.79 | 4.15 | 3.58 | 3.94 | 0.321 | 0.31 | 0.54 | 1.00 |
| | 3 | 3.77 | 3.08 | 3.54 | 3.10 | 0.197 | 0.01 | 0.46 | 0.37 |
| A:P | 0 | 4.74 | 4.68 | 3.97 | 4.41 | 0.221 | 0.39 | 0.04 | 0.26 |
| | 3 | 4.48 | 5.03 | 3.52 | 4.79 | 0.233 | <0.01 | 0.01 | 0.09 |
| (A+B):P | 0 | 5.43 | 5.44 | 4.48 | 5.06 | 0.230 | 0.20 | 0.02 | 0.22 |
| | 3 | 5.14 | 5.62 | 4.07 | 5.34 | 0.278 | <0.01 | 0.01 | 0.08 |
| $NH_3$-N, m*M* | 0 | 5.84 | 6.79 | 4.87 | 6.68 | 0.555 | 0.04 | 0.34 | 0.44 |
| | 3 | 15.11 | 14.34 | 16.15 | 14.35 | 0.932 | 0.22 | 0.59 | 0.60 |
| Total lactate, m*M* | 0 | 0.56 | 0.65 | 0.57 | 0.65 | 0.039 | 0.06 | 0.81 | 0.97 |
| | 3 | 0.83 | 0.71 | 0.78 | 0.68 | 0.107 | 0.24 | 0.69 | 0.91 |
| Nitrate, mg/L[5] | 0 | <LoQ | <LoQ | <LoQ | <LoQ | -- | -- | -- | -- |
| | 3 | <LoQ | <LoQ | <LoQ | <LoQ | -- | -- | -- | -- |
| Nitrite, mg/L | 0 | 0.12 | 0.58 | 0.12 | 0.83 | 0.246 | 0.07 | 0.66 | 0.66 |
| | 3 | 0.24 | 0.45 | 0.24 | 0.37 | 0.168 | 0.32 | 0.79 | 0.79 |
| pH | Mean | 6.20 | 6.30 | 6.07 | 6.42 | 0.101 | 0.03 | 0.94 | 0.15 |
| Hydrogen, µ*M* | Mean | 3.58 | 45.28 | 4.03 | 21.00 | 14.097 | 0.07 | 0.41 | 0.39 |

[1] Data were collected during 2 non-consecutive days in wk 4.

[2] CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

[3] Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.

[4] Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.

[5] LoQ = Limit of Quantification = 13.3 mg/L or 0.22 m*M*.

The diet LIN decreased (*P* = 0.03) total protozoa concentration in the rumen before feeding whereas NIT did not affect this population. The toxic effect of linseed towards protozoa was not observed when associated with nitrate (*P* = 0.02; Table 4). Compared with CON, diet LIN reduced total protozoa concentration by specifically acting on entodiniomorphs (-52%).

Inversely, diet NIT tended to increase ($P$ = 0.09) large entodiniomorphs and increased ($P$ = 0.02) *Isotricha* before feeding.

**Table 4** Rumen protozoa populations of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

| Item[1] | Time after feeding (h) | Diet[2] | | | | SEM | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CON | NIT | LIN | LIN+NIT | | Nitrate | Linseed | Linseed × nitrate |
| Total protozoa, $log_{10}$/mL | 0 | 5.87 | 5.71 | 5.55 | 5.73 | 0.060 | 0.91 | 0.03 | 0.02 |
| | 3 | 5.71 | 5.49 | 5.37 | 5.58 | 0.080 | 0.95 | 0.14 | 0.03 |
| Entodiniomorphs, $log_{10}$/mL | | | | | | | | | |
| Small | 0 | 5.86 | 5.68 | 5.54 | 5.71 | 0.057 | 0.95 | 0.03 | 0.02 |
| (<100 µm) | 3 | 5.69 | 5.46 | 5.36 | 5.56 | 0.080 | 0.86 | 0.16 | 0.03 |
| Large | 0 | 4.09 | 4.18 | 3.66 | 4.01 | 0.110 | 0.09 | 0.03 | 0.29 |
| (>100 µm) | 3 | 3.97 | 4.00 | 3.62 | 3.97 | 0.109 | 0.14 | 0.13 | 0.18 |
| Holotrichs, $log_{10}$/mL | | | | | | | | | |
| *Dasytricha* | 0 | 3.51 | 3.65 | 2.67 | 3.58 | 0.497 | 0.29 | 0.35 | 0.42 |
| (<100 µm) | 3 | 3.49 | 3.78 | 2.75 | 3.69 | 0.521 | 0.23 | 0.40 | 0.51 |
| *Isotricha* | 0 | 1.90 | 3.19 | 2.29 | 3.11 | 0.484 | 0.02 | 0.63 | 0.47 |
| (>100 µm) | 3 | 2.88 | 3.25 | 2.53 | 2.89 | 0.494 | 0.42 | 0.42 | 1.00 |

[1] Data were collected during 2 non-consecutive days in wk 4.

[2] CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

[3] Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.

*Diet digestibility and nitrogen balance*

Daily DM and OM intake were not affected by treatments and averaged 12.4 kg DMI/d (Table 5). Fiber intake was reduced with linseed-containing diets ($P$ < 0.01) compared with other diets. Linseed associated with nitrate had a similar reducing effect towards fiber intake. Total tract digestibility of DM and OM was not affected by diets and linseed supplemented alone or in association with nitrate tended to reduce ($P$ < 0.10) fiber digestibility.

Total N losses (% of N intake) were greater for diets including linseed compared with other diets ($P$ = 0.03) leading to lesser N retention for LIN and LIN+NIT ($P$ = 0.03; Table 6). This was not related to differences in daily fecal N losses between diets, but to numerically greater urinary N losses with linseed-containing diets ($P$ = 0.08).

**Table 5** Daily nutrient intake and total tract digestibility of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

| Item[1] | Diet[2] | | | | SEM | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|---|
| | CON | NIT | LIN | LIN+NIT | | Nitrate | Linseed | Linseed × nitrate |
| Daily nutrient intake, kg/d | | | | | | | | |
| DM | 12.4 | 12.3 | 12.5 | 12.3 | 0.55 | 0.09 | 0.73 | 0.51 |
| OM | 11.4 | 11.3 | 11.4 | 11.3 | 0.51 | 0.14 | 0.74 | 0.45 |
| NDF | 5.0 | 5.0 | 4.8 | 4.7 | 0.22 | 0.08 | <0.01 | 0.41 |
| ADF | 2.9 | 2.9 | 2.8 | 2.7 | 0.13 | 0.05 | <0.01 | 0.76 |
| GE intake, MJ/d | 216.8 | 205.1 | 228.5 | 217.2 | 9.67 | <0.01 | <0.01 | 0.88 |
| Total tract digestibility, % | | | | | | | | |
| DM | 63.7 | 64.1 | 64.0 | 63.3 | 0.77 | 0.85 | 0.65 | 0.43 |
| OM | 68.1 | 68.5 | 68.3 | 67.9 | 0.64 | 0.98 | 0.76 | 0.50 |
| NDF | 44.8 | 45.2 | 44.2 | 40.1 | 1.58 | 0.22 | 0.07 | 0.14 |
| ADF | 44.5 | 45.1 | 42.9 | 38.4 | 2.11 | 0.31 | 0.06 | 0.20 |

[1] Data were collected during 6 consecutive days in wk 4.

[2] CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

[3] Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.

**Table 6** Nitrogen balance of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

| Item[1] | Diet[2] | | | | SEM | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|---|
| | CON | NIT | LIN | LIN+NIT | | Nitrate | Linseed | Linseed × nitrate |
| N intake, g/d | 252.5 | 242.5 | 242.5 | 227.5 | 11.59 | <0.01 | <0.01 | 0.13 |
| Fecal N losses | | | | | | | | |
| g/d | 101.6 | 95.4 | 94.8 | 96.8 | 4.28 | 0.47 | 0.37 | 0.18 |
| As % of N intake | 40.1 | 39.4 | 39.5 | 42.5 | 1.18 | 0.27 | 0.25 | 0.10 |
| Urinary N losses | | | | | | | | |
| g/d | 133.1 | 117.7 | 135.8 | 120.2 | 6.13 | 0.02 | 0.61 | 0.99 |
| As % of N intake | 52.5 | 48.8 | 56.5 | 52.7 | 1.82 | 0.09 | 0.08 | 0.97 |
| Total N losses | | | | | | | | |
| g/d | 234.7 | 213.0 | 230.6 | 217.0 | 9.12 | 0.01 | 0.99 | 0.45 |
| As % of N intake | 92.6 | 88.3 | 96.0 | 95.2 | 2.25 | 0.20 | 0.03 | 0.35 |
| N retained | | | | | | | | |
| g/d | 18.5 | 28.3 | 10.7 | 11.8 | 5.82 | 0.26 | 0.03 | 0.36 |
| As % of N intake | 7.4 | 11.8 | 4.0 | 4.8 | 2.25 | 0.20 | 0.03 | 0.35 |

[1] Data were collected during 6 consecutive days in wk 4.

[2] CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

[3] Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.

## Discussion

*Effect of nitrate on cows' health*

In the rumen, nitrate is converted to nitrite and then ammonia. While nitrate is non-toxic, nitrite can be poisonous for the animal. If nitrite accumulates in the rumen, it can pass through the rumen wall into the blood and convert Hb to metHb, which cannot then transport oxygen to the tissues (Lewis, 1951). The level of blood metHb determines the severity of symptoms, which are brown mucous membrane discoloration, depressed feed intake and animal performances, and even coma and death in extreme cases (Bruning-Fann and Kaneene, 1993). Throughout this experiment, animals were unaffected by nitrate supplementation, as shown by the BW gain, the constant intake, and the low rumen concentrations of nitrate and nitrite and blood metHb. Nitrate feeding requires precise management of its distribution and careful control of animal health status. To deal with these issues, the use of slow-release encapsulated nitrate was shown to be effective at mitigating $CH_4$ emissions of lambs (3.4% nitrate in DM, inducing a 9.7% $CH_4$ reduction per percent added nitrate; El-Zaiat et al., 2014) or beef heifers

(2.3% nitrate in DM, inducing a 8.0% $CH_4$ reduction per percent added nitrate; Lee et al., 2014a, b) without raising blood metHb levels.

*Methane emissions*

We observed that supplying 2.6% added fat from linseed oil reduced $CH_4$ (g/kg DMI) by 17%, corresponding to a 6.5% reduction in $CH_4$ per percentage unit of added lipids from linseed. This result is in the range of previous meta-analysis data reporting that $CH_4$ (g/kg DMI) is reduced by 4.4% per percentage unit of fat (irrespective of lipid source) added to diet (Grainger and Beauchemin, 2011) or by 5.6% per percentage unit of linolenic acid from linseed (Doreau et al., 2011). Conversely, Veneman et al. (2013) did not explain the absence of any $CH_4$-mitigative effect (g/kg DMI, g/kg milk) of a similar level of linseed oil in lactating cows.

Nitrate fed alone reduced $CH_4$ (g/kg DMI) by 22%, corresponding to a 9.8% reduction per percentage unit of nitrate fed. This result is in the range of previous experimental data reporting a $CH_4$ (g/kg DMI) reduction of between 7.9 and 12.2% per percentage unit of added nitrate in the diet of sheep (Nolan et al., 2010; Van Zijderveld et al., 2010) or cattle (Van Zijderveld et al., 2011; Hulshof et al., 2012; Veneman et al., 2013). The $CH_4$-mitigating effect of nitrate is consequently greatly repeatable whatever the diet and the ruminant species.

The association of nitrate and linseed oil reduced $CH_4$ (g/kg DMI) by 32%. This result showed for the first time that there is a positive and additive effect between nitrate and linseed oil on methanogenesis. Theoretically, as these dietary strategies have different mechanisms of action, $CH_4$ reduction should reach 39% for a fully additive effect. Several reasons may explain the difference between theoretical and observed $CH_4$ reduction. First, we suggest that linseed reduced $H_2$ production and that nitrate only acted on this reduced $H_2$ pool. Then, according to stoichiometry and considering that control $CH_4$ emissions is equal to 100, $CH_4$ emissions corrected for the $CH_4$-mitigating effect of LIN (17%) would be $100 - 100 \times 0.17 = 83$. These $CH_4$ emissions corrected for the $CH_4$-mitigating effect of NIT (22%) would be $83 - 83 \times 0.22 = 65$. In total, this corresponds to an expected $CH_4$ reduction of 35% with LIN+NIT, which is close to the observed level of $CH_4$ reduction. In addition, LIN+NIT had lesser FA content compared with LIN, which may be linked to unnoticed pellets manufacturing issues. Knowing that 1% added fat from linseed reduced $CH_4$ by 6.5%, the difference in FA content between LIN+NIT (1.0% added fat) and LIN (2.6% added fat) corresponded to a $CH_4$ mitigation potential of 10.4%, suggesting a fully additive effect

between linseed oil and nitrate. At least, the formation of calcium salts via the reaction between lipids and soluble calcium from calcium nitrate may reduce the additive effects of LIN+NIT (Keyser et al., 1985).

The association of nitrate and linseed oil appears interesting: this same level of $CH_4$ reduction with linseed oil or nitrate fed individually could not be achieved without greater risks of metHb for nitrate or lesser diet digestibility for linseed oil. Other kinds of antimethanogenic combinations have shown various interactions. Tea saponin and soybean oil reduced $CH_4$ (g/kg DMI) from lambs by 27% and 14%, respectively, when distributed alone and by 19% when fed in association (Mao et al., 2010). Again in lambs, $CH_4$ (g/kg DMI) was reduced by 25% by chestnut tannin, 14% by coconut oil and 33% by the association chestnut tannin plus coconut oil (Liu et al., 2011). A fully additive effect was observed with two $H_2$-sink products fed to lambs, with a $CH_4$ reduction of 32% with nitrate, 16% with sulfate and 47% with nitrate plus sulfate (Van Zijderveld et al., 2010).

*Mechanisms of $CH_4$ reduction: focus on rumen fermentation parameters*

The reduction in $CH_4$ emissions observed in this trial did not cause a rumen dysfunction, as VFA concentration was not affected by diet and pH was only marginally modified. Two factors may explain the $CH_4$-mitigating effect of linseed oil. On the one hand, lipids from linseed oil half-reduced the rumen concentration of protozoa, although not as strongly as in previous experiments testing similar levels of lipids (-82% in a silage-based diet, Chung et al., 2011; -84% in a concentrate-rich hay-based diet, Ueda et al., 2003). The anti-protozoal effect of linseed combined with nitrate was less evident, probably because of the lesser fat content in LIN+NIT compared with LIN. Protozoa are known to be important $H_2$ producers via their hydrogenosomes (Morgavi et al., 2012) and their reduction is often associated with a decrease in methanogenesis (Guyader et al., 2014). Consequently, in this study, linseed supplementation reduced $H_2$ production, but as dissolved $H_2$ concentrations in the rumen were not affected by lipids, we assume that methanogens also used less $H_2$. On the other hand, linseed oil increased propionate proportion which is a $H_2$-consuming pathway competing with methanogenesis (Newbold et al., 2005). Most literature reports do not show an effect of linseed on rumen VFA composition (Chung et al., 2011; Doreau et al., 2009; Martin et al., 2011). To a minor extent, $H_2$ may have been consumed during PUFA biohydrogenation, but this pathway would deviate only 1 to 2.6% of ruminal $H_2$ (Czerkawski, 1986). The lesser $CH_4$

emissions throughout the day from LIN cows compared with CON cows indicated that linseed oil continuously modified rumen fermentation and microbial parameters.

Nitrate is an electron acceptor in several anaerobic environments. Its $CH_4$-mitigating effect is assumed to be related to a reduction of $H_2$ availability for methanogens due to its reduction to nitrite and ammonia (Ungerfeld and Kohn, 2006). To our knowledge, ours is the first study to report a post-feeding pattern of dissolved $H_2$ concentrations in the rumen. The CON and LIN diets presented stable and low rumen $H_2$ concentrations (3.8 μ*M* on average), which are in the range of concentrations (0.1 to 50 μ*M*) given by a literature review (Janssen, 2010). However, adding nitrate to the diet with or without linseed oil induced a peak in rumen dissolved $H_2$ concentrations up to 2 h post-feeding (up to 88 μ*M* on average), coinciding with a drop in $CH_4$ emissions and a rise of gaseous $H_2$ (measured in wk 5 of the last two experimental periods; data not shown) as already reported by Van Zijderveld et al. (2011). In presence of nitrate, the excess of dissolved $H_2$ further released in belched gas means that $H_2$ was produced at a greater rate than it was utilized. This may result from a toxic effect of nitrate (Van Zijderveld et al., 2010) or nitrite (Iwamoto et al., 2001) on $H_2$-users such as methanogens. This putative action is transient, lasting for 3 h post-feeding, as shown by the increase in $CH_4$ emissions from nitrate-fed cows up to levels similar to control-diet cows.

*Diet digestibility and nitrogen balance*

Supplying diets with linseed oil (2.6% added fat) did not affect total tract digestibility of DM and OM but tended to reduce total tract fiber digestibility to a same extent when fed alone or in association with nitrate. This result is not consistent with a previous study on lambs supplemented with crude linseed (2.4% added fat; Machmüller et al., 2000). These different results may be explained by the forms of linseed which affect availability of lipids supply: linseed oil would have a more negative effect on total tract digestibility than extruded and crude linseed (Martin et al., 2008). Adding 3% calcium nitrate as a substitute for urea did not reduce total tract digestibility confirming previous experiments on sheep fed hay and 4% potassium nitrate (Nolan et al., 2010) and on dairy cows fed maize silage and 2.8% calcium nitrate (Van Zijderveld et al., 2011). Nitrate neither affected N retention nor the distribution of N losses between urine and feces. Similar results were obtained with dairy cows (2.6% nitrate; Van Zijderveld et al., 2011), steers (2.3% nitrate; Lee et al., 2014a) and lambs (2.3% nitrate; Li et al., 2012) fed isonitrogenous diets, showing that nitrate can substitute urea as a source of non-protein N.

The association of nitrate and linseed oil is an efficient strategy to decrease $CH_4$ yields in non-lactating cows without altering diet digestibility. Linseed oil supplementation reduced $CH_4$ emissions throughout the day, while nitrate had a transient but marked action from when fed up to 3 h post-feeding. Methane production was further reduced when both linseed and nitrate were fed in association. Linseed oil reduced $H_2$-producers like protozoa, whereas nitrate acted as a $H_2$-sink and may have inhibited rumen $H_2$-users, as suggested by the rise of dissolved $H_2$ concentrations with this dietary treatment. Further work to characterize the quantity, activity and diversity of rumen microbiota should clarify the mechanisms behind the effects of these dietary treatments. In addition, it will be necessary to assess the long-term $CH_4$-mitigative effect of linseed oil associated with nitrate on farmed ruminants. Finally, the effect of nitrate on animal performances and the absence of residues in ruminant end-products still need further research.

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# STEP 2: Long-term methane mitigating effect of linseed plus nitrate supplemented to dairy cows

## Objective

1/ To evaluate the long-term effect of association of feeding strategies acting on $H_2$ production (lipids from linseed, toxic effect towards protozoa) and $H_2$ utilization (nitrate from calcium nitrate, $H_2$-sink through nitrate reduction to nitrite and ammonia) on $CH_4$ emissions, lactating performances of dairy cows and animal health (blood metHb, nitrate and nitrite residues in milk and processed milk products).

2/ To check the effect of linseed plus nitrate on total tract digestibility, N balance and rumen fermentation after long-term supplementation.

## Experimental approach

16 lactating cows → 8 animals → **CON**: 54% corn silage + 6% hay + 40% pelleted concentrate

→ 8 animals → **LIN+NIT**: CON + 1.8% nitrate (from calcium nitrate) + 3.5% added lipids (from extruded linseed)

17 weeks of experiment (wk 1 to 3 = Adaptation; wk 4 to 17 = Measurement)

| WEEK | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Daily intake and milk yield<br>Milk composition (once a week) | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ |
| Nitrate and nitrite residues in milk and milk products (once a week) | | ■ | | | | ■ | | | | ■ | | | | ■ |
| Blood metHb (3.5 h after morning feeding, once a week) | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ |
| Daily kinetics of $CH_4$ emissions (2 days) | | ■ | | | | | | | | | | | ■ | |
| Total tract digestibility, N balance (5 days)<br>Rumen fermentation (3.5 h after morning feeding, once a week) | | | | | | | | | | | | | | ■ |

## Main results

- Throughout the experiment, intake and milk production tended to be lower for dairy cows supplemented with LIN+NIT, but feed efficiency was similar between diets.
- From wk 4 to 17, average metHb level was 1.2%. No additional nitrate and nitrite residues were detected in milk and processed milk products from cows fed LIN+NIT.
- Diet LIN+NIT reduced $CH_4$ emissions by 29%, with a persistent effect throughout the 4 months of the experiment.
- Digestibility of nutrients and N balance were similar between diets. Diet LIN+NIT reduced total VFA concentration and increased C2/C3 ratio and protozoa concentration postfeeding.

## Conclusion

The association of linseed plus nitrate is an efficient and long-term $CH_4$-mitigating strategy, which does not alter diet digestibility, N efficiency or animal health. However, the energetic benefits of the decreased $CH_4$ emissions did not appear beneficial for the animal.

# Long-term methane-mitigating effect of linseed plus nitrate supplemented to dairy cows

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**Abstract**

The objective of this experiment was to study the long-term effect of linseed plus nitrate on $CH_4$ emission and performance in dairy cows. We also assessed the effect of this feeding strategy on the presence of nitrate and nitrite residues in milk products, total tract apparent digestibility, N balance and rumen fermentation. Sixteen lactating Holstein cows were allocated to 2 groups in a randomized design conducted in parallel for 17 weeks. Diets were (dry matter basis): 1) control (54% corn silage, 6% hay, and 40% concentrate) or 2) control plus 3.5% added fat from linseed and 1.8% nitrate (LIN+NIT). Diets were equivalent in terms of crude protein (16%), starch (28%), and neutral detergent fiber (33%), and were offered twice daily. Cows were fed ad libitum, except during wk 5, 16, and 17 in which feed was restricted to 95% of dry matter intake (DMI) to ensure complete consumption of meals. Milk production and DMI were measured weekly. Nitrate and nitrite concentration in milk and milk products was determined monthly. Daily methane emissions were quantified in open chambers (wk 5 and 16). Total tract apparent digestibility, N balance, and rumen fermentation parameters were determined at the end of the experiment (wk 17). Daily DMI tended to be lower with LIN+NIT from wk 4 to 16 (-5.1 kg/d on average). The LIN+NIT diet decreased milk production during 6 non-consecutive weeks (-2.5 kg/d on average). Nitrate or nitrite residues were not detected in milk and associated products of cows fed either diet. The LIN+NIT diet reduced $CH_4$ emissions to a similar extent at the beginning (wk 5) and end (wk 16) of the trial: CH4 reduction averaged 46% (g/d), 29% (g/kg DMI), and 35% (g/kg milk). Both diets did not affect N efficiency and nutrients apparent digestibility. In the rumen, LIN+NIT did not affect protozoa number but reduced total volatile fatty acid concentration by 12% and propionate concentration by 31%. We concluded that linseed plus nitrate has a long-term methane-reducing effect in dairy cows. We also found a concomitant negative effect on milk production, despite a similar feed efficiency between diets. Further work is required to optimize the doses of linseed plus nitrate to avoid reduced cows performance. The consumption of milk products from animals fed nitrate is safe for human consumption in terms of nitrate and nitrite residues.

**Keywords:** linseed plus nitrate, long-term, methane, milk product, ruminant

**Introduction**

Linseed and nitrate are both proven dietary strategies for reducing $CH_4$ emissions from ruminants (Gerber et al., 2013). If used extensively, they could significantly abate enteric $CH_4$ emissions at a national scale (Doreau et al., 2014). However, the combination of these two feeding strategies on $CH_4$ production has not been studied before. In a short-term experiment on non-lactating cows, we reported that the combination of linseed oil (4% of DM) plus nitrate (2.25% of DM) reduced methanogenesis by 32% without affecting apparent diet digestibility. Compared to linseed oil and nitrate fed individually, the effect of this combination on $CH_4$ production was additive (Guyader et al., 2014a), because these two dietary strategies share different modes of action in the rumen. Polyunsaturated lipids from linseed are thought to act as inhibitors of $H_2$-producers such as protozoa (Guyader et al., 2014a), whereas nitrate is thought to act as a $H_2$-sink, competing with methanogenesis. Nitrate and nitrite are also toxic to methanogens (Guyader et al., 2014c).

In-practice, the use of these strategies at farm scale requires further investigation into their potential long-term effects. Linseed (3% added lipids) had a persistent $CH_4$-mitigating effect on dairy cows for up to 1 yr (Martin et al., 2011). The long-term $CH_4$-mitigating effect of nitrate (2.1% of DM) fed over 3 mo has been demonstrated in dairy cows (Van Zijderveld et al., 2011). However, the long-term $CH_4$-reducing effect of dietary linseed plus nitrate has not been tested.

Another issue to assess before practical application of linseed plus nitrate as an animal nutrition strategy is the potential for adverse effects of nitrate supplementation on human and animal health. To our knowledge, the effect of dietary nitrate on milk quality, including the absence of nitrate and nitrite residues in milk, has not been tested, whereas excess nitrite from nitrate reduction in the mouth may promote gastric irritation in humans (Weitzberg and Lundberg, 2013). One study did show an absence of additional nitrate and nitrite residues in meat when lambs were fed 3.4% encapsulated nitrate (El-Zaiat et al., 2013). Nitrate may also alter animal health by increasing the concentration of blood methemoglobin (metHb; Lewis, 1951). Without adaptation (Lee and Beauchemin, 2014), nitrite from nitrate reduction can accumulate in the rumen, passing through the blood and leading to subclinical methemoglobinemia (30-40% of metHb; Bruning-Fann and Kaneene, 1993).

The main objective of this experiment was to investigate the long-term effect of linseed plus nitrate on $CH_4$ emissions and lactation performance in dairy cows. As a secondary objective, nitrate metabolism was assessed by measuring metHb levels in blood and nitrate and nitrite

levels in milk and processed milk products. We also evaluated the effect of linseed plus nitrate on total tract apparent digestibility, N balance, and rumen fermentation parameters at the end of the experiment.

**Materials and methods**

The experiment was conducted at the UERT experimental dairy cow facilities at the INRA's Saint-Genès-Champanelle-based research centre in France from January to May 2014. All procedures involving animals were performed in accordance with French Ministry of Agriculture guidelines for animal research, and all applicable European guidelines and regulations on animal experimentation (http://www2.vet-lyon.fr/ens/expa/acc_regl.html).

*Animals, Diets and Feeding*

Sixteen lactating (including 7 primiparous) Holstein cows were used. At the start of the experiment, cows had an average milk yield of 33.4 ± 7.1 kg/d at 61 ± 23 DIM, and an average BW of 706 ± 67 kg. The experiment was conducted for 17 wk as a randomized block design where cows were separated into 2 groups balanced for calving date and milk production. Cows were housed in a freestall barn except during the 2 measurement periods (wk 5 and wk 16-17 for $CH_4$ and digestibility measurements) in which they were housed individually.

The first group of cows (n = 8 of which 4 primiparous) was fed the control diet (CON), and the second group of cows (n = 8 of which 3 primiparous) was fed CON with 9.8% extruded linseed and 2.4% calcium ammonium nitrate (75% $NO_3$ in DM) on a DM basis (LIN+NIT). The doses of extruded linseed and nitrate were estimated to reduce $CH_4$ emission by 10 to 15% when fed alone (Doreau et al., 2014) and by 20 to 30% when fed together. Diets were formulated to meet the requirements of lactating dairy cows (30 kg daily milk production without BW change) and to be equivalent in terms of CP, gross energy (GE) and starch content (INRA, 2010; Table 1). On a DM basis, diets were composed of 54% corn silage, 6% natural grassland hay, and 40% concentrate given as pellets (InVivo NSA, Longué Jumelles, France).

**Table 1** Ingredients and chemical composition of the experimental diets

| | Diet[1] | |
|---|---|---|
| Item | CON | LIN+NIT |
| Ingredients, % of DM | | |
| Corn silage[2] | 54.00 | 54.00 |
| Hay | 6.00 | 6.00 |
| Pelleted concentrate | | |
| Corn | 11.88 | 12.00 |
| Barley | 3.36 | 2.52 |
| Soybean meal | 5.24 | 1.28 |
| Rapeseed meal | 2.00 | 3.12 |
| Sunflower meal | 0.00 | 0.80 |
| Extruded linseed[3] | 0.00 | 9.80 |
| Soybean hulls | 6.60 | 2.00 |
| Wheat bran | 6.00 | 4.20 |
| Dehydrated beet pulp | 0.94 | 0.00 |
| Calcium ammonium nitrate[4] | 0.00 | 2.40 |
| Urea | 0.80 | 0.00 |
| Calcium carbonate | 1.13 | 0.00 |
| Dicalcium phosphate | 0.44 | 0.26 |
| Beet molasses | 1.20 | 1.20 |
| Mineral-vitamin premix | 0.20 | 0.20 |
| Sodium chloride | 0.17 | 0.18 |
| Fungicide | 0.02 | 0.02 |
| Flavoring[5] | 0.02 | 0.02 |
| Chemical composition[6], % of DM | | |
| OM | 93.06 | 93.50 |
| CP | 15.81 | 15.59 |
| NDF | 34.74 | 31.91 |
| ADF | 18.20 | 16.58 |
| Starch | 27.98 | 28.78 |
| Ether extract, % of DM | 3.23 | 6.75 |
| Total fatty acid, % of DM | 2.54 | 5.86 |
| Gross energy, MJ/kg of DM | 17.64 | 18.37 |
| FA profile, % of total FA | | |
| C16:0 | 16.87 | 13.89 |
| C18:0 | 2.40 | 2.74 |
| C18:1 n-9 | 25.06 | 23.34 |
| C18:2 n-6 | 43.24 | 31.59 |
| C18:3 n-3 | 9.06 | 25.05 |

[1] CON = diet control; LIN+NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis.

[2] Fermentation characteristics of fresh silage juice: pH = 3.57; Acetic acid = 0.74 g/100g; Lactic acid = 3.01 g/100g; N-$NH_3$ = 0.02 g/100g.

[3] Extruded linseed, InVivo NSA, Longué Jumelles, France

[4] Calcium ammonium nitrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$; Phytosem, Pont-du-Château, France) contained 75% $NO_3$ on a DM basis.

[5] Gusti, Nutriad, Chester, England.

[6] Average of chemical composition from samples (n = 3) taken in wk 5, 16 and 17.

Two weeks before starting the experiment, all cows were fed CON diet ad libitum. Then, LIN+NIT-group animals were diet-adapted by progressively replacing CON concentrate with LIN+NIT concentrate over a 2-wk adaptation period to achieve the dose of 2.4% calcium ammonium nitrate at the beginning of wk 3. Hay was offered once daily (0800 h) and corn silage mixed with concentrates was offered twice daily (66% at 0930 h and 34% at 1600 h). All cows were fed ad libitum except during measurement weeks in which offered feed was restricted to 95% of individual voluntary feed intake to ensure complete consumption of the diet. Forage-to-concentrate ratio was kept as close as possible to the target ratio by adjusting the amounts of offered feed every week based on quantity and composition of the refusals of the previous week. Cows had free access to water throughout the experiment.

*Measurements and Analyses*

*Liveweight and Blood Methemoglobin.* Animals were weighed the week before starting the experiment (wk 0) then in wk 5, 10, 14, and 20. Blood metHb levels were measured 3.5 h after morning feeding on cows fed LIN+NIT and compared with levels of control samples taken on these same animals in wk 0. Blood was then sampled twice a week from wk 1 to wk 3 (adaptation to nitrate) and once a week from wk 4 to the end of the experiment (wk 17). Blood (10 mL) was sampled from the tail vein into K2-EDTA collection tubes (Venosafe, Terumo, Guyancourt, France) then carried on ice to the nearest hospital (CHU Gabriel Montpied, Clermont-Ferrand, France) to determine metHb concentrations by spectrophotometry within 1 h (UV-160, Shimadzu, Marne-La-Vallée, France; Kaplan, 1965).

*Intake.* Offered feed and refusals were weighed and recorded daily throughout the experiment. During the 2 measurement periods (wk 5 and wk 16-17), samples (200 g) of hay and concentrates were taken once a week, and samples (200 g) of corn silage were taken twice a week. For each feed sample, one aliquot was used to determine DM content (103°C for 24 h) and the other aliquot was stored at 4°C (hay and concentrates) or -20°C (corn silage) until analysis of chemical composition. Refusals were measured for DM when they exceeded 2 kg/d per animal during measurement weeks. Composition of refusals was identified as forage (hay, corn silage) or concentrate, and their chemical composition was considered similar to that of feed.

Chemical composition analyses were carried out on fresh (hay, concentrates) or freeze-dried (corn silage) feedstuff samples after grinding (1 mm) (InVivo Labs, Chierry, France). Organic

matter was determined by ashing at 550°C for 6 h (method 942.05; AOAC, 2005). Total N was analyzed by combustion according to the Dumas method (method 968.06; AOAC, 2005), and CP content was calculated as N × 6.25. Fiber (NDF and ADF) was determined by sequential procedures (Van Soest et al., 1991) after pretreatment with amylase and sulfuric acid, and was expressed exclusive of residual ash. Starch was analyzed using an enzymatic method (Faisant et al., 1995), and gross energy was analyzed by adiabatic bomb calorimetry (C200 model, IKA, Staufen, Germany). Ether extract was determined after acid hydrolysis (method 954.02; AOAC, 2005), and FA composition was determined by gas chromatography of methyl esters (method 969.33; AOAC, 2005). Juice from fresh corn silage was obtained by maceration to analyze pH, N-$NH_3$ (Kjeldahl method, method 2001.11; AOAC, 2005), acetic and lactic acid (gas chromatography with a flame ionization detector) concentrations (InVivo Labs, Chierry, France).

*Methane Emissions.* Daily total $CH_4$ emissions were measured continuously using 4 open chambers (1 animal per chamber) in wk 5 and 16. Each animal spent 2 consecutive days (48 h) in a chamber to measure the $CH_4$ emissions of the 8 animals from a same group within the week. Animals were allocated to the same chamber for both measurement periods.

The chambers (16.6 $m^3$) were made of steel uprights with clear polycarbonate walls allowing sight contact between animals and with the farm personnel. They operated at a slight negative pressure, with an airflow oscillating between 700 and 800 $m^3/h$ (approximately 45 air renewals per hour). Airflow entered the chamber through an aperture at the bottom of the rear door (0.42 $m^2$), and exited through an exhaust duct situated at the top of the chamber, over the head of the animal. Airflow in the exhaust duct of each chamber was continuously measured (CP300, KIMO, Montpon-Ménestérol, France), and recorded once every 5 min (KT-210-AO, KIMO, Montpon-Ménestérol, France). Concentration of gases in the barn and in the 4 chambers was alternatively analyzed at a 0.1 Hz sample frequency for 5 min every 25 min using an infrared detector (Ultramat 6, Siemens, Karlsruhe, Germany) and recorded (Nanodac Invensys, Eurotherm Automation SAS, Dardilly, France). Gas concentrations between 2 measurement intervals in the barn and in the chambers were estimated by linear regression. The detector was manually calibrated the day before each measurement week using pure $N_2$ and a mixture of $CH_4$ (650 ppm) and $CO_2$ (700 ppm) in $N_2$.

Chamber rear doors were opened twice daily: in the morning for milking and to remove feces and urine, and in the afternoon for milking. Chamber front doors were opened 3 times a day

for feeding. Front and rear doors were not simultaneously opened in order to avoid an air stream into the chamber. In total, the doors of each chamber were opened for 30 min per 24 h. Data collected while doors were open were deleted and a proportional calculation was applied to recover 24-h $CH_4$ emissions.

*Diet Apparent Digestibility and Nitrogen Balance.* Total tract apparent digestibility and N balance were determined from total and separate collection of feces and urine for 5 d during wk 17. To separate urine from feces, cows were fitted with flexible tubes connected to a 30-L flask containing 500 mL of 3 *M* sulfuric acid to achieve a urine pH lower than 3 and thereby avoid N volatilization. Feces and urine were removed once daily.

Every day, after weighing and mixing of feces, a 1% fresh aliquot was used to determine DM (103°C for 24 h), and another 1% fresh aliquot was pooled across days for each animal and frozen (-20°C). At the end of the experiment, pooled samples were thawed, freeze-dried, and ground (1 mm) to determine OM, N, NDF, and ADF content as previously described for feed (InVivo Labs, Chierry, France).

For urine, every day after weighing, a 1% fresh aliquot was pooled across days for each animal and frozen (-20°C). At the end of the experiment, after thawing, the N content of urine was determined by the Kjeldahl method (method 2001.11; AOAC, 2005 ; InVivo Labs, Chierry, France).

*Milk Yield and Composition.* Throughout the experiment, milk yield was determined daily. For determination of milk composition (fat, protein, lactose, and urea concentration), individual milk samples (30 mL) mixed with potassium bichromate (Merck, Fontenay-Sous-Bois, France) were taken and stored at 4°C before analysis within 2 d (Galilait, Theix, France). Samples were taken at morning and afternoon milking 2 d per week when animals were in the $CH_4$ chambers (wk 5 and 16). Milk fat, protein, and lactose content were analyzed by infrared spectrometry with a 3-channel spectrophotometer (MilkoScan, Foss Electric, Hillerod, Denmark; method 972.16; AOAC, 1990). Milk urea concentration was analyzed by the dimethylamino-4-benzaldehyde colorimetric method (Potts, 1967).

For analysis of nitrate and nitrite residues in individual milk, samples (300 mL) from the morning milking were taken once a week in wk 5, 9, 13, and 17. For analysis of nitrate and nitrite residues in pooled milk and milk products, the morning milk of all animals was pooled by diet in wk 9 and 17. Pooled milk was sampled (100 mL) and local farmhouse-style

products were made (yoghurts, whey, curd and 6-wk ripened Saint-Nectaire cheese). All samples were stored at 4°C before analysis within 2 d (Eurofins Analytics, Nantes, France). Nitrate and nitrite residues in individual milk samples were analyzed by ion chromatography (method 993.30; AOAC, 1990) with a limit of quantification (LoQ) of 10 mg/kg for nitrate and 5 mg/kg for nitrite. In pooled milk samples and processed milk products, nitrate and nitrite residues were analyzed by spectrometry after nitrate reduction with cadmium (ISO 14673; ISO, 2004) with a LoQ of 5 mg/kg for nitrate and 0.5 mg/kg for nitrite.

*Rumen Fermentation Parameters.* On the last day of wk 17, rumen samples were collected 3.5 h after the morning feeding by stomach tubing (Shen et al., 2012). Samples were strained through a polyester monofilament fabric (250 μm pore size) and the filtrate was subsampled for VFA and $NH_3$ concentration analyses and protozoa counting. For VFA analysis, 0.8 mL of filtrate was mixed with 0.5 mL of a 0.5 *M* HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid. For $NH_3$ analysis, 1 mL of filtrate was mixed with 0.1 mL of 5% orthophosphoric acid. These samples were stored at -20°C until analysis. For protozoa counting, 2 mL of filtrate was mixed with 2 mL of methyl green-formalin saline solution, and stored at room temperature in the dark until counting.

Concentrations of VFA and $NH_3$ were analyzed by gas chromatography with a flame ionization detector and by colorimetry, respectively (Morgavi et al., 2008). Protozoa were counted by microscopy, and categorized as either small (< 100 μm) or large (> 100 μm) entodiniomorphs, or as holotrichs (*Dasytricha* or *Isotricha*) (Williams and Coleman, 1992). Data for protozoa were $log_{10}$-transformed before statistical analysis.

*Statistical Analyses*

Data were analyzed using the MIXED procedure of SAS (Version 9.4; SAS Institute, 2009). All statistical models included the animal nested within diet as random effect.

Data collected throughout the experiment (intake, milk production and composition) or on two occasions ($CH_4$ emissions) were averaged per week as there was no statistical difference between days within a week. The statistical model included diet (n = 2), week (n = 17 for intake and milk and n = 2 for $CH_4$), and diet × week interaction as fixed effects. Week was treated as a repeated measurement. For intake, milk production and composition (except for urea), data collected the week before starting the experiment (wk 0) were used as covariates. For continuous measures of $CH_4$ emissions, the model included diet (n = 2), week (n = 2),

hour (n = 24), diet × week and diet × hour interactions as fixed effects. Hour was treated as a repeated measurement. As the interaction diet × week was not statistically significant, averaged data of the two weeks are presented in Figure 4. For the repeated measurements, several covariance structures were tested (variance component, autoregressive, compound symmetry, unstructured, and toeplitz) and structure with the lowest Akaike's information criteria was chosen. Then, variance component was always used as covariance structure, except for daily $CH_4$ emissions where compound symmetry was used.

Data collected at the end of the experiment (apparent digestibility, N balance, rumen fermentation and microbial parameters) were analyzed with diet (n = 2) as fixed factor.

Differences between diets were considered significant at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.1$. Least squares means are reported throughout.

## Results

*Liveweight and Blood Methemoglobin*

During the 17-wk experiment, cows fed CON or LIN+NIT lost on average 32 and 22 kg to reach a final BW of 697 ± 62 kg and 662 ± 67 kg, respectively. During the 3-wk period of adaptation to nitrate, the maximum metHb level was 13.0% (Figure 1). From wk 4 to wk 17, average metHb level was 1.2%. Maximum metHb level peaked at 30.8% for one cow in wk 17, whereas average metHb level for all other cows on that week averaged 4.4%.

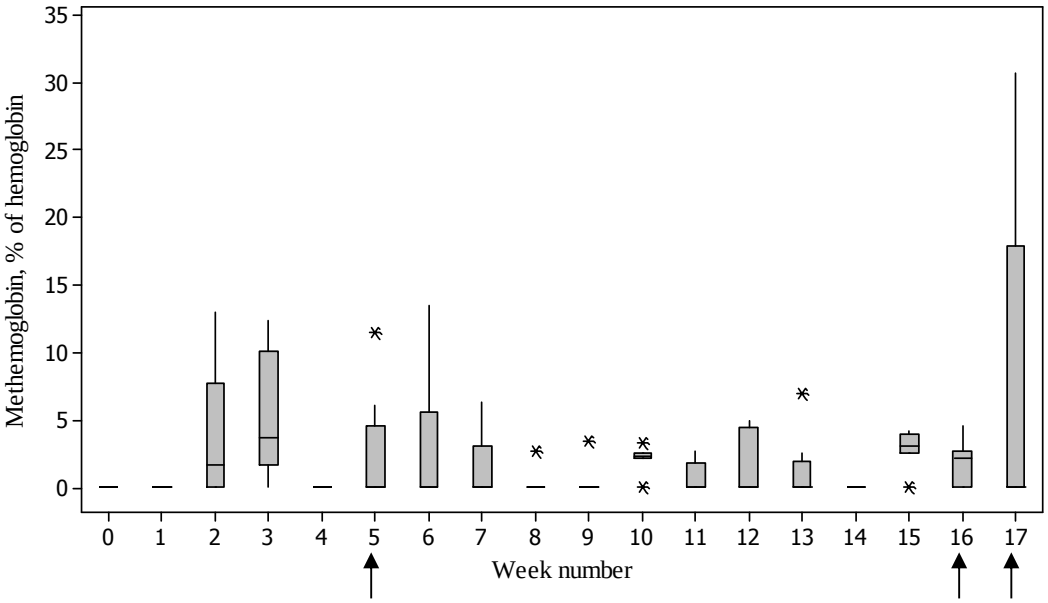


**Figure 1** Boxplot of blood metHb levels of lactating cows fed 10% extruded linseed plus 1.8% nitrate (n = 8) during 17 weeks. In wk 0, animals were fed a control diet. Linseed and nitrate were firstly incorporated in wk 1. Blood was analyzed in wk 0 and then twice a week during wk 1, 2 and 3 and once a week from wk 4 to 17. The box represents the quartiles with the median within the box, and the vertical lines represent the maximum and minimum value within 1.5 interquartile range of the higher and lower quartile, respectively. Values greater than 1.5 interquartile range are considered as outliers and are identified with a star. The arrows indicate the measurement weeks.

*Intake and Milk Yield*

Daily DMI was similar between diets in wk 1, 2, 3, and 17 (Figure 2) and tended to be lower with LIN+NIT from wk 4 to 16 (-5.1 kg/d on average; $P \leq 0.10$). This tendency between diets was also observed for DM and OM intake ($P = 0.070$ and $P = 0.078$, respectively) when cows were in chambers for 2 d for $CH_4$ measurements (wk 5 and 16; Table 2). Fiber intakes were lower with LIN+NIT ($P = 0.008$ for NDF and $P = 0.007$ for ADF) whereas dietary treatments did not affect gross energy intake (Table 2).

We found no between-diet difference in milk production over two thirds of the experiment (11 wk out of 17; Figure 3), whereas in wk 4, 5, 7, 9, 10, and 17, milk production was lower with LIN+NIT (-2.5 kg/d on average; $P \leq 0.05$). During the 2 d in chambers (wk 5 and 16), cows fed LIN+NIT also tended to produce less milk (-2.8 kg/d on average; $P = 0.078$; Table 2). Feed efficiency was similar between diets in wk 5 and tended to be higher for LIN+NIT in wk 16 (diet × week, $P = 0.079$; Table 2).

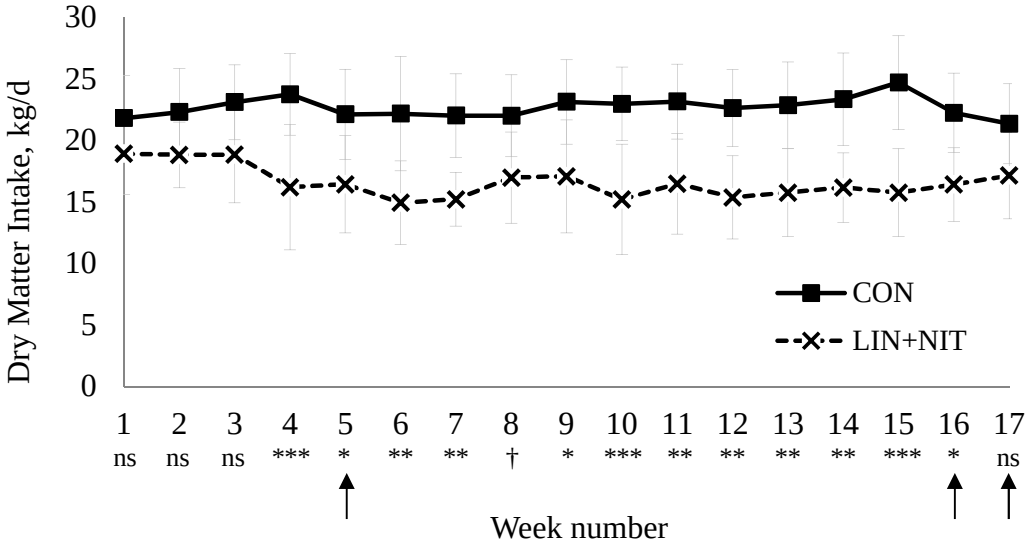


**Figure 2** Dry matter intake of lactating cows fed a control diet (CON; n = 8) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN+NIT; n = 8) during 17 weeks (averages of 4 days per week). Errors bars indicate SD. Symbols indicate weekly statistical comparison between CON and LIN+NIT (†$P \leq 0.10$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Arrows indicate measurement weeks.

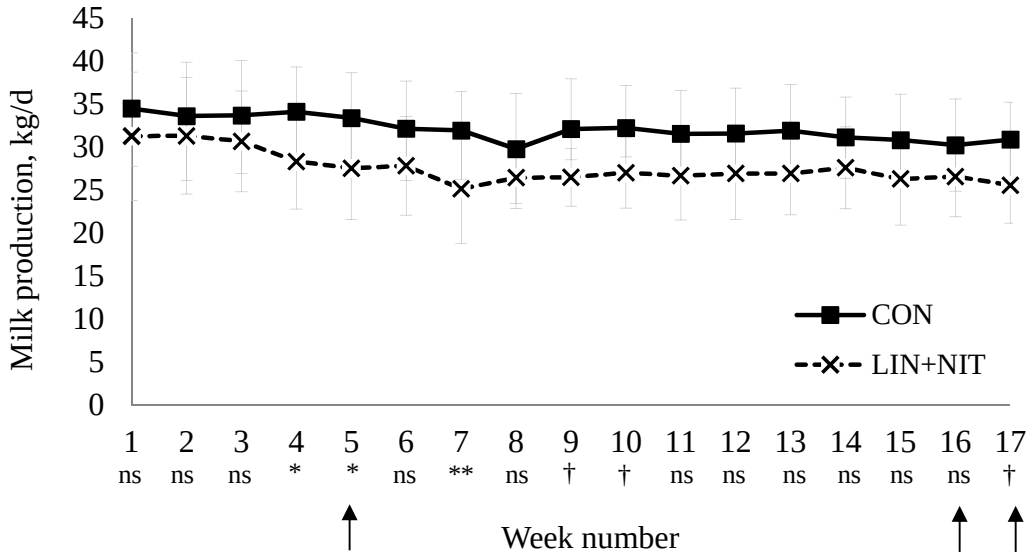


**Figure 3** Milk yield of lactating cows fed a control diet (CON; n = 8) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN+NIT; n = 8) during 17 weeks (averages of 4 days per week). Errors bars indicate SD. Symbols indicate weekly statistical comparison between CON and LIN+NIT (†$P \leq 0.10$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Arrows indicate measurement weeks.

**Table 2** Daily nutrient intake, milk yield and composition, and methane emissions of lactating cows fed a control diet (n = 8) or a diet supplemented with a combination of linseed and nitrate (n = 8)

| | Diet[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Item[2] | CON | | LIN+NIT | | | P-value | | |
| Week number[3] | 5 | 16 | 5 | 16 | SEM | Diet | Week | Diet × Week |
| Nutrient intake | | | | | | | | |
| DM, kg/d | 20.8 | 20.7 | 18.8 | 17.3 | 1.00 | 0.070 | 0.182 | 0.293 |
| OM, kg/d | 19.4 | 19.2 | 17.6 | 16.2 | 0.93 | 0.078 | 0.183 | 0.292 |
| NDF, kg/d | 7.25 | 7.19 | 6.03 | 5.55 | 0.338 | 0.008 | 0.204 | 0.326 |
| ADF, kg/d | 3.80 | 3.80 | 3.13 | 2.88 | 0.177 | 0.007 | 0.205 | 0.319 |
| Gross energy, MJ/d | 367.5 | 364.3 | 345.3 | 318.0 | 17.82 | 0.183 | 0.172 | 0.276 |
| Milk yield and composition | | | | | | | | |
| Milk yield, kg/d | 32.6 | 29.9 | 28.9 | 28.1 | 1.05 | 0.078 | 0.001 | 0.052 |
| Feed efficiency[4], kg milk/kg DMI | 1.57 | 1.46 | 1.58 | 1.67 | 0.064 | 0.148 | 0.888 | 0.079 |
| Fat, g/d | 1393.1 | 1205.7 | 1030.3 | 1075.1 | 91.67 | 0.060 | 0.198 | 0.045 |
| Protein, g/d | 1031.0 | 996.9 | 851.4 | 865.3 | 45.24 | 0.026 | 0.615 | 0.243 |
| Lactose, g/d | 1654.6 | 1501.8 | 1489.3 | 1365.5 | 54.60 | 0.060 | <0.001 | 0.608 |
| Urea, g/d | 7.5 | 6.1 | 2.4 | 2.0 | 0.55 | <0.001 | 0.061 | 0.223 |
| Fat, g/kg | 41.9 | 39.1 | 36.5 | 39.1 | 2.23 | 0.298 | 0.961 | 0.185 |
| Protein, g/kg | 31.5 | 33.2 | 29.4 | 30.9 | 0.78 | 0.045 | 0.009 | 0.902 |
| Lactose, g/kg | 50.7 | 50.1 | 51.9 | 48.8 | 0.72 | 0.948 | 0.002 | 0.027 |
| Urea, mg/dL | 22.2 | 19.4 | 8.7 | 7.7 | 1.51 | <0.001 | 0.216 | 0.524 |
| Methane emission | | | | | | | | |
| g $CH_4$/d | 470.6 | 459.1 | 254.0 | 247.6 | 34.13 | <0.001 | 0.640 | 0.895 |
| g $CH_4$/kg DM intake | 21.5 | 20.8 | 14.6 | 15.3 | 1.30 | 0.003 | 1.000 | 0.310 |
| g $CH_4$/kg milk | 14.0 | 14.8 | 9.4 | 9.3 | 1.02 | 0.002 | 0.560 | 0.516 |
| % of gross energy intake | 6.1 | 5.9 | 4.0 | 4.2 | 0.36 | 0.001 | 0.988 | 0.307 |

[1] CON = diet control; LIN+NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis. [2] Average of 2 d in chambers in wk 5 and 16. For intake, milk yield and composition, a covariate (data obtained in wk 0) was included in the statistical model. [3] Number of weeks of distribution of dietary treatment. [4] Feed efficiency = milk yield/DMI.

In chambers, milk fat and lactose concentrations were similar between diets, whereas LIN+NIT reduced milk protein ($P = 0.045$) and urea ($P < 0.001$) contents by 6.8% and 60.6%, respectively. For both diets, nitrate and nitrite concentrations in individual milk samples, pooled milk samples, and milk products were lower than the LoQ, except for curd from CON in wk 17 and cheese from CON and LIN+NIT in wk 9 in which low nitrite concentrations were detected (1.5 mg/kg).

*Methane Emissions*

Diet LIN+NIT reduced $CH_4$ emissions by 29.3% when expressed in grams per kilogram of DMI ($P = 0.003$), and by 35.1% when expressed in grams per kilogram of milk ($P = 0.002$). Whatever the mode of expression of $CH_4$ emission, there was no significant effect of week or diet × week interaction (Table 2). This shows that $CH_4$ emissions of CON and LIN+NIT were similar between the 2 wk of measurements, and that the difference between diets was repeatable, even after 11 wk of dietary treatments.

Methane emissions for a 24-h period, averaged for the 2 wk of measurements, are presented in Figure 4. Methane emissions were similar between diets during the 4 h preceding the morning feeding, then LIN+NIT reduced $CH_4$ emissions for the first 12 h after the morning meal ($P \leq 0.05$).

*Diet Apparent Digestibility and Nitrogen Balance*

Apparent digestibility of DM, OM, and NDF was similar between diets, and averaged 67.5, 69.4, and 50.6%, respectively (Table 3). The LIN+NIT diet tended to reduce ADF (-3.8%; $P = 0.070$) and CP (-2.9%; $P = 0.074$) apparent digestibility.

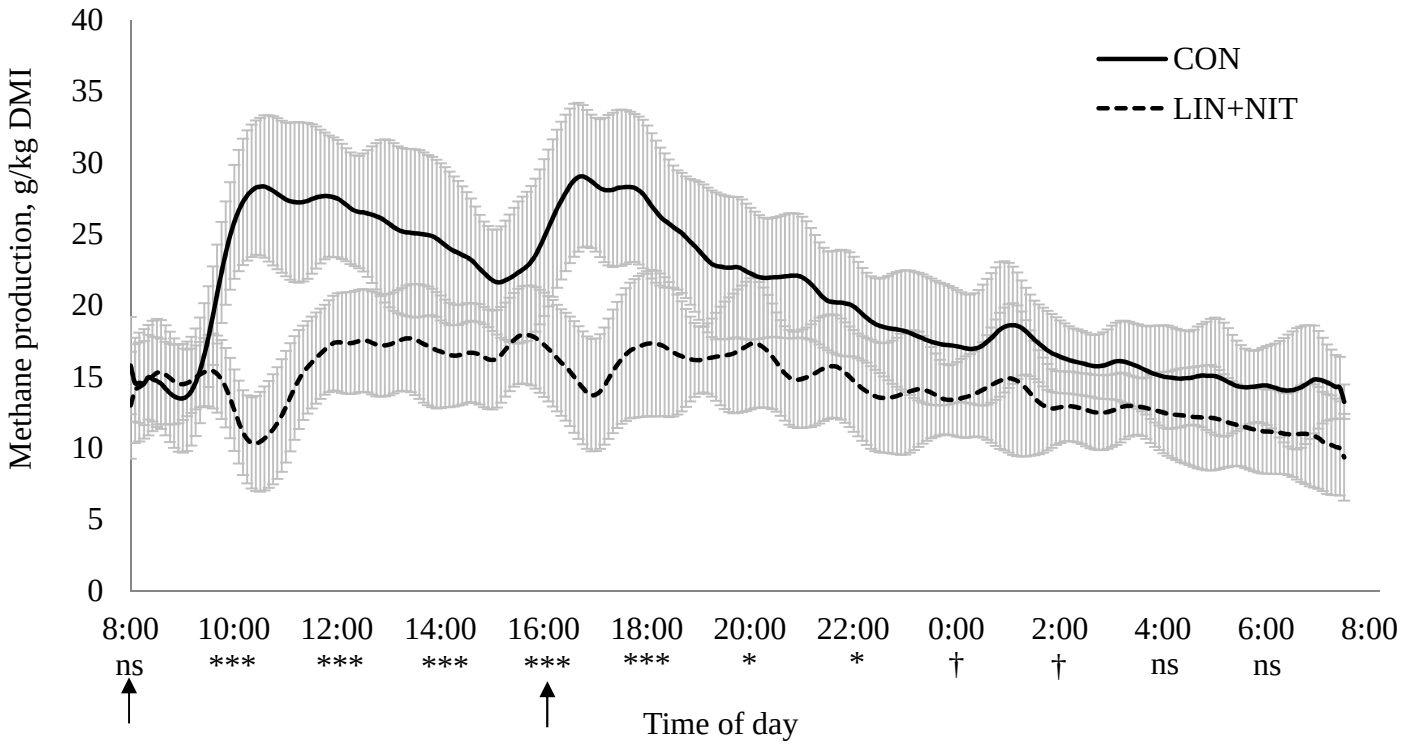


**Figure 4** Daily $CH_4$ production pattern of lactating cows fed a control diet (CON; n = 8) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN+NIT; n = 8) during 17 weeks (raw data; averages of 2 days and 2 weeks of $CH_4$ measurement; wk 5 and 16). Errors bars indicate SD. Symbols indicate hourly statistical comparison between CON and LIN+NIT (†$P \leq 0.10$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Arrows indicate time of feeding.

Nitrogen intake was 22% lower with LIN+NIT ($P$ = 0.001). Consequently, LIN+NIT led to lower fecal N losses, urinary N losses, and N retained in milk ($P$ = 0.016, $P$ < 0.001, and $P$ = 0.003, respectively). However, N distribution was unaffected by diet. On average for both diets, 35.7% ($P$ = 0.074), 24.1% ($P$ = 0.071), and 29.9% ($P$ = 0.937) of N intake was directed towards feces, urine, and milk, respectively. Finally, N balance was positive and similar between diets and averaged 52.6 g/d or 10.5% of N intake.

**Table 3** Total tract apparent digestibility and nitrogen balance of lactating cows after 17 weeks feeding a control diet (n = 8) or a diet supplemented with a combination of linseed and nitrate (n = 8)

| | Diet[1] | | | |
|---|---|---|---|---|
| Item[2] | CON | LIN+NIT | SEM | P-value |
| Total tract apparent digestibility, % | | | | |
| DM | 67.8 | 67.2 | 0.74 | 0.531 |
| OM | 69.8 | 69.0 | 0.73 | 0.458 |
| NDF | 51.3 | 49.9 | 1.11 | 0.393 |
| ADF | 47.5 | 43.7 | 1.35 | 0.070 |
| CP | 65.8 | 62.9 | 1.05 | 0.074 |
| Starch | 98.5 | 97.9 | 0.24 | 0.109 |
| N intake, g/d | 548.1 | 425.1 | 21.56 | 0.001 |
| Fecal N losses | | | | |
| g/d | 187.3 | 156.8 | 7.88 | 0.016 |
| % of N intake | 34.2 | 37.1 | 1.05 | 0.074 |
| Urinary N losses | | | | |
| g/d | 138.2 | 96.1 | 5.74 | <0.001 |
| % of N intake | 25.4 | 22.7 | 0.98 | 0.071 |
| Total fecal and urinary N losses | | | | |
| g/d | 325.5 | 253.0 | 11.16 | <0.001 |
| % of N intake | 59.6 | 59.7 | 1.36 | 0.939 |
| Milk N output | | | | |
| g/d[3] | 163.5 | 126.1 | 7.24 | 0.003 |
| % of N intake | 29.9 | 29.8 | 1.05 | 0.937 |
| N Balance[4] | | | | |
| g/d | 59.1 | 46.1 | 9.87 | 0.365 |
| % of N intake | 10.5 | 10.5 | 1.73 | 0.990 |

[1] CON = diet control; LIN+NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis.

[2] Average of 5 d of total tract apparent digestibility and N balance measurement in wk 17. No covariate was included in the statistical model.

[3] Milk N output = (milk yield × milk protein concentration)/average N content in milk (6.38 g N/g milk protein).

[4] N balance = N intake - total fecal and urinary N losses - milk N output.

*Rumen Fermentation and Microbial Parameters*

Concentration of $NH_3$ in the rumen did not change with diets (Table 4). Diet LIN+NIT reduced total VFA (-12 m*M*; *P* = 0.020) and propionate concentrations (-8 m*M*; *P* = 0.003) without affecting acetate and butyrate concentrations. These differences in VFA profile induced an increase in C2/C3 and C2+C4/C3 ratios (*P* = 0.003) with LIN+NIT.

Total concentration of protozoa in the rumen tended to increase with LIN+NIT (+53%; *P* = 0.052). This was linked to a higher concentration of small entodiniomorphs and *Dasytricha* (*P* = 0.047 and *P* = 0.014, respectively). Concentrations of large entodiniomorphs and *Isotricha* were unaffected by diets.

**Table 4** Fermentation parameters and protozoal concentration in the rumen of lactating cows after 17 weeks feeding a control diet (n = 8) or a diet supplemented with a combination of linseed and nitrate (n = 8)

| | Diet[1] | | | |
|---|---|---|---|---|
| Item[2] | CON | LIN+NIT | SEM | *P*-value |
| $NH_3$, m*M* | 10.14 | 10.97 | 1.648 | 0.736 |
| VFA concentration, m*M* | | | | |
| Total VFA | 104.1 | 91.7 | 3.35 | 0.020 |
| Acetate (C2) | 58.6 | 56.9 | 1.95 | 0.561 |
| Propionate (C3) | 25.6 | 17.6 | 1.65 | 0.003 |
| Butyrate (C4) | 15.2 | 14.1 | 1.61 | 0.635 |
| Minor VFA[3] | 4.71 | 3.08 | 0.577 | 0.055 |
| C2/C3 | 2.36 | 3.27 | 0.170 | 0.003 |
| (C2+C4)/C3 | 2.99 | 4.08 | 0.213 | 0.003 |
| Total protozoa, $log_{10}$/mL | 5.03 | 5.32 | 0.095 | 0.052 |
| Entodiniomorphs, $log_{10}$/mL | | | | |
| Small (< 100 µm) | 5.01 | 5.31 | 0.095 | 0.047 |
| Large (> 100 µm) | 3.39 | 3.11 | 0.217 | 0.387 |
| Holotrichs, $log_{10}$/mL | | | | |
| *Dasytricha* (< 100 µm) | 2.22 | 3.02 | 0.191 | 0.014 |
| *Isotricha* (> 100 µm) | 3.24 | 2.48 | 0.115 | 0.140 |

[1] CON = diet control; LIN+NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis.

[2] Data from rumen samples taken the last day of wk 17. No covariate was included in the statistical model.

[3] Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.

## Discussion

*Intake, Milk Production, and Nitrogen Balance*

Throughout the experiment, intake and milk production tended to be lower for dairy cows supplemented with LIN+NIT. As feed efficiency (kg of milk per kg of feed) was similar between diets, the lower intake may explain the lower milk production. The lower intake with LIN+NIT is difficult to explain because diets had similar net energy content. In addition, in a short-term experiment, intake was similar between non-lactating cows fed with or without linseed plus nitrate (Guyader et al., 2014a). Individual nitrate supplementation at higher doses than here (1.8%) did not reduce intake of restricted-fed dairy cows (2.1%, Van Zijderveld et al., 2011; 2.0%, Veneman et al., 2014) and sheep (2.5%, Nolan et al., 2010; 2.6%, Van Zijderveld et al., 2010) but tended to reduce DMI of dairy cows (2.0%, Veneman et al., 2014) and steers (2.3%, Hulshof et al., 2012) fed ad libitum. Linseed applied at doses higher than here (3.5% added fat) did not have a negative effect on the intake or milk production of dairy cows (5.1% added fat, Ferlay et al., 2013; 4% added fat, Veneman et al., 2014) fed ad libitum or restricted. One study reported a lower DMI (-7%) by lactating cows fed a grass silage-based diet supplemented with linseed (3% added fat; Martin et al., 2011). The only study that simultaneously used linseed plus nitrate (4% added fat plus 2.3% nitrate) on cows did not result in intake changes, but the cows were non-lactating and not fed ad libitum (Guyader et al., 2014a). Consequently, we hypothesize that LIN+NIT fed together ad libitum may have an inhibitory effect on voluntary intake linked to a tendency for lower ADF apparent digestibility. Earlier reviews have highlighted the negative correlation between fiber digestibility and voluntary intake through a lower passage rate of particles from the rumen and greater rumen filling (Allen, 1996). Further work would help determine the optimal quantity of dietary LIN+NIT that can be provided without reducing intake, which is also an essential step towards making this feeding strategy acceptable at the farm scale.

The LIN+NIT diet had no effect on concentration and production of fat and lactose. This result confirms previous experiments on dairy cows supplemented with nitrate (2.1% nitrate in a corn silage based diet; Van Zijderveld et al., 2011) or with incremental amounts of extruded linseed (up to 5.1% added fat in hay- or corn silage-based diets; Ferlay et al., 2013). The LIN+NIT diet reduced milk protein concentration by 7% (-2.2 g/kg milk) and milk protein production by 15% (-155.6 g/d). In dairy cows fed 2.1% nitrate, Van Zijderveld et al. (2011) also reported reduced milk protein concentrations (-5% or -1.4 g/kg of milk) but no

effect on milk protein production whereas milk yield was stable. The reduced milk protein content may not be linked to linseed supplementation, as milk protein content of dairy cows was not affected by 3.5% added fat from extruded linseed in hay- or corn silage-based diets (Ferlay et al., 2013).

Nitrogen balance was positive for both diets, even if it may be overestimated because volatile N losses from faeces and urine, dermal and scurf N losses were not taken into account (Spanghero and Kowalski, 1997). Nevertheless, N balance was similar between diets with the same N distribution between milk, feces and urine. In addition, average N efficiency (N in milk/N intake) was similar between CON and LIN+NIT (30%) and close to the data given in the literature (25%, with a range between 15 and 40%; Calsamiglia et al., 2010). This result shows that dairy cows use nitrate in the same way as they use other N sources. With LIN+NIT, milk urea concentration and production were 12.6 mg/dL and 4.6 g/d less, respectively, than CON. This marked decrease was surprising and in contradiction with previous experiments on dairy cows showing no effect of extruded linseed (1.1% added fat; Pezzi et al., 2007) or nitrate (2.1% nitrate; Van Zijderveld et al., 2011) on milk urea content. We assumed that the between-diet difference in milk urea comes from the lower N intake of animals fed LIN+NIT, as N intake is known to correlate positively with milk urea (Spek et al., 2013).

The main concern when using nitrate in animal nutrition is its potential negative effect on animal and human health. To avoid increase of blood metHb in animals (Lewis, 1951), progressive adaptation to nitrate is essential (Lee and Beauchemin, 2014). In addition, it is important that nitrate is homogenously incorporated in the ration, not top dressed, to avoid swift ingestion of the daily dose. By applying these recommendations, we did not observe rises in metHb levels in animals fed LIN+NIT, similarly to a previous experiment on dairy cows fed 2.1% nitrate (Van Zijderveld et al., 2011). However, we cannot explain the greater metHb level observed in the last week of the experiment. In terms of human health, nitrate and nitrite are common food additives used for their anti-bacterial properties against lethal pathogens (European Food Safety Authority, 2009). However, an excess of nitrite from nitrate reduction in the mouth may promote gastric inflammation (Weitzberg and Lundberg, 2013). Regulations have been adopted to keep concentrations of nitrate and nitrite residues within recommended daily allowances for nitrate and nitrite intake (3.75 and 0.13 mg/kg BW per day, respectively; European Food Safety Authority, 2009), and Europe has limited nitrate concentration in drinking water to 50 mg/L (Benjamin, 2000). Nitrate intake mainly comes

from vegetables (60 to 80%), water (15 to 20%) and animal-based products (10 to 15%), while 80 to 85% of nitrite exposure comes from conversion of nitrate in the mouth. Vegetables such as spinach can contain up to 1,614 mg nitrate per kg. Here, nitrate and nitrite residues in milk or milk products were lower than the LoQ of the technique (5 mg/kg for nitrate and 0.5 mg/kg for nitrite), except in cheese from CON and LIN+NIT (1.5 mg/kg nitrite). These novel data confirm previous work on lamb meat (El-Zaiat et al., 2013), and show that animals can metabolize nitrate and nitrite without transferring residues into animal products. Consequently, long-term supplementation with nitrate (4 months) can be safely proposed in ruminant nutrition without risks for human health, as a $CH_4$-mitigating strategy and a source of non-protein nitrogen to replace urea.

*Methane Emissions and Associated Digestive Mechanisms*

In our experiment using open chambers, $CH_4$ emissions of dairy cows fed CON averaged 21.2 g/kg DMI. This value is close to the estimate calculated by an equation based on OM content of the diet and OM digestibility (21.4 g/kg DMI; Sauvant et al., 2011), and is also in accordance with the average $CH_4$ emission of cattle fed diets without supplementation of $CH_4$-mitigating treatments (20.7 g/kg DMI, number of treatments = 33) as compiled from a database used for a previous meta-analysis (Guyader et al., 2014b).

The reduction in $CH_4$ emission (g/kg of DMI) averaged 29% when dairy cows were supplemented with 1.8% nitrate plus 3.5% added fat from extruded linseed, corresponding to our expected theoretical $CH_4$ reduction. This confirms our previous results obtained on non-lactating cows supplemented with 2.2% nitrate plus 4% added fat from linseed oil (Guyader et al., 2014a) and shows that LIN+NIT can efficiently reduce $CH_4$ emissions regardless of the physiological stage of cows. We also observed a severe $CH_4$-mitigating effect of LIN+NIT just after feeding, which was most probably linked to the effect of nitrate quickly metabolized in the rumen. This result agrees with previous studies (Van Zijderveld et al., 2010; Guyader et al., 2014a). Methane reduction with LIN+NIT corresponds to a saving of 2% of gross energy intake, without positive responses on apparent digestibility, weight gain or body condition score (data not shown) of the animals. The absence of relationship between $CH_4$ reductions and dairy cow performance has also been reported previously (Van Zijderveld et al., 2011).

The $CH_4$-mitigating effect of LIN+NIT was maintained throughout the 4 months of the experiment, indicating that this dietary strategy could be applied on farms. The long-term $CH_4$-mitigating effect of nitrate (2.1%) and extruded linseed (2.5% added fat) fed individually

to dairy cows was also maintained during 3 mo (Van Zijderveld et al., 2011) and 1 yr (Martin et al., 2011), respectively.

The LIN+NIT diet did not change rumen protozoa concentration as previously observed with non-lactating cows supplemented with 2.2% nitrate plus 4% added fat from linseed (Guyader et al., 2014a). Diet LIN+NIT increased the acetate/propionate and (acetate+butyrate)/propionate ratios due to a decrease in ruminal propionate which is normally a competitive pathway of methanogenesis (Martin et al., 2010). This contrasts with our previous work in which LIN+NIT did not change rumen fermentation parameters (Guyader et al., 2014a). However, in the present work, the relationship between $CH_4$ production and rumen fermentation and microbial parameters should be interpreted with caution given the large differences in time scale between $CH_4$ measurement periods and rumen samplings through stomach tubing. Consequently, the $CH_4$-mitigating effect of LIN+NIT would not be explained by a reduction in acetate and butyrate synthesis, nor by a reduction in protozoa which are important $H_2$-producers. Other mechanisms must be involved in the $CH_4$-mitigating effect of LIN+NIT. Both supplements may act as $H_2$ sinks. Based on stoichiometric calculation and assuming complete reduction of nitrate to nitrite and ammonia, and complete biohydrogenation of polyunsaturated fatty acids, the reduction of 1 mol nitrate reduces $CH_4$ by 1 mol, and the biohydrogenation of 1 mol C18:1, C18:2, and C18:3 reduces $CH_4$ by 0.25, 0.50 and 0.75 mol, respectively. Extending this calculation here, 325.8 g/d of nitrate ingested by dairy cows would have reduced $CH_4$ by 5.25 mol/d (or 90.1 g/d) and 600.9 g of fatty acid ingested by dairy cows (23, 32, and 25% of C18:1, C18:2, and C18:3, respectively) would have reduced $CH_4$ by 0.87 mol/d (or 14.9 g/d). In total, $H_2$ consumption by LIN+NIT would have reduced $CH_4$ emissions by 105.0 g/d, explaining 49% of the observed $CH_4$ reduction. The remaining decrease can thus be explained by non-stoichiometric processes. The LIN+NIT diet may also act on rumen microbiota. Previous work showed that nitrate reduced both quantity (2.6% nitrate to sheep, Van Zijderveld et al., 2010) and activity (2.3% nitrate to non-lactating cows, Guyader et al., 2014c) of methanogens. The anti-methanogenic effect of polyunsaturated fatty acid has also been demonstrated in pure culture of methanogens (Prins et al., 1972) and in previous experiments with cattle (4% added fat, Guyader et al., 2014c; 3.5% added fat, C. Martin, unpublished data). In addition, $H_2$ production must have been lowered with LIN+NIT owing to a lower quantity of fermentable substrates in the rumen (lower DMI, quantity of carbohydrates due to lipids substitution and fiber digestibility) which directly reduced $CH_4$ emissions.

**Conclusions**

The association of linseed plus nitrate is an efficient feeding strategy to reduce $CH_4$ emissions in the long-term without altering diet apparent digestibility, N efficiency or animal health. However, the energetic benefits of the decreased $CH_4$ emissions to the animals were not observed. Additional data is needed on changes in rumen microbiota in order to fully understand the $CH_4$-mitigating effect of the association of linseed plus nitrate. Moreover, to make this dietary strategy acceptable by farmers, further work is required to optimize the doses of linseed plus nitrate in an effort to avoid concomitant reduction in intake and milk production. A life cycle assessment will also be needed to evaluate the environmental benefit and economic cost of this dietary strategy in order to raise the prospects of using this strategy at farm level.

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# STEP 3: Absence of methane mitigating effect of tea saponin fed to non-lactating and lactating cows

## Objective

1/ To test the effect of a different feeding strategy acting on $H_2$ production (saponin from tea, toxic effect towards protozoa) on $CH_4$ emissions and associated ruminal mechanisms of non-lactating and lactating cows.
2/ To assess its effect on diet digestibility, N balance and lactating performances.

## Experimental approach

Trial **1**

4 non-lactating cows → 2 × 2 Factorial design →

**CON-1**: 50% hay + 50% pelleted concentrate
**NIT-1**: CON + 2.3% nitrate (from calcium nitrate)
**TEA-1**: CON + 0.5% saponin (from tea)
**TEA+NIT-1**: CON + 0.5% saponin + 2.3% nitrate


4 experimental periods of 5 weeks (wk 1 to 3 = Adaptation; wk 4 to 5 = Measurement)

| WEEK | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Daily intake | ■ | ■ | ■ | ■ | ■ |
| Blood metHb (3 h after morning feeding, once a week) | ■ | ■ | ■ | | ■ |
| Total tract digestibility, N balance (6 days)<br>Rumen fermentation (3 h after morning feeding, twice a week) | | | | | ■ |
| Daily kinetics of $CH_4$ emissions (4 days) | | | | ■ | |

## Experimental approach

Trial **2**

8 lactating cows → 2 × 2 Crossover design →

**CON-2**: 54% corn silage + 6% hay + 40% pelleted concentrate
**TEA-2**: CON + 0.5% saponin (from tea)

4 experimental periods of 5 weeks (wk 1 to 3 = Adaptation; wk 4 to 5 = Measurement)

| WEEK | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Daily intake, milk yield | ■ | ■ | ■ | ■ | ■ |
| Total tract digestibility, N balance<br>Rumen fermentation (3.5 h after morning feeding, once a week) | | | | | ■ |
| Daily kinetics of $CH_4$ emissions (2 days)<br>Milk composition (once a week) | | | | ■ | |

## Main results

Trial **1** Trial **2**

- Intake tended to be reduced by tea saponin (-12% in trial 2). Milk yield was reduced by 18% without modification in its composition.
- Methane emissions from non-lactating and lactating cows were unaffected by tea saponin. This plant extract also poorly modified rumen fermentation parameters.
- Tea saponin did not affect N balance but tended to improve fiber digestibility in both experiments.

## Conclusion

Tea saponin tended to reduce zootechnical performances of cattle, without reducing their $CH_4$ emissions whatever the physiological stage. We assume that the active compound of the plant was degraded during the pelleting process. This plant extract tended to increase fiber digestibility of lactating cows, without affecting N balance. Further work is required to improve tea saponin palatability and to confirm its positive effect on fiber digestibility.

# Absence of methane-mitigating effect of tea saponin fed to non-lactating and lactating cows

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*In progress*

**Abstract**

Two *in vivo* trials were conducted to study the effect of tea saponin alone (or in association with nitrate) on methane emissions and digestive processes in cows. Trial 1 was designed as a 2 × 2 factorial design on four rumen cannulated non-lactating cows fed four diets: 1/ control (CON-1) consisting of hay and concentrate (50:50 on a DM basis), 2/ control with 0.5% tea saponin (TEA-1), 3/ control with 2.3% nitrate (NIT-1) and 4/ control with 0.5% tea saponin and 2.3% nitrate (TEA+NIT-1). Trial 2 was carried out on eight lactating cows fed two diets in a 2 × 2 crossover design: 1/ control (CON-2) consisting of maize silage, hay and concentrate (54:6:40 on a DM basis) and 2/ control with 0.5% tea saponin (TEA-2). In both trials, each experimental period lasted five weeks including two last weeks of measurement during which animals were restricted fed between 90-95% of *ad libitum* intake. Intake and milk production were daily measured all along trials. Daily methane emissions were quantified using open chambers, total tract digestibility and nitrogen balance were determined from total feces and urine collected separately, rumen fermentation parameters and protozoal concentration were analyzed from samples taken after morning feeding. In both trials, tea saponin tended to reduce DM intake (-12% in trial 2). Milk production was reduced (-18%) with TEA-2, most likely because of the tendency for lower intake as feed efficiency was similar between diets. Methane emissions (g/kg dry matter intake) were similar between CON-1 and TEA-1, and were reduced to the same extent with NIT-1 and TEA+NIT-1 (-28% on average). On dairy cows, methane emissions (g/kg dry matter intake) were increased by 14% with TEA-2. Total tract digestibility and nitrogen balance were similar among diets in the two trials, except for ADF digestibility which tended to be improved with TEA-2 (+8%). Ruminal fermentative parameters (ammonia, lactate, and volatile fatty acids ratios) were poorly changed by diets: we observed an increase of acetate and a decrease of butyrate with nitrate-containing diets in trial 1, and an increase of acetate with tea saponin in trial 2. Whatever trial, protozoa concentrations were similar among diets. We conclude that tea saponin was not efficient to reduce methane emissions from cattle in our experimental conditions. Further work is required to confirm positive effect of this plant extract on fiber digestibility.

**Keywords:** cattle, digestibility, methane, nitrate, tea saponin

**Implications**

The use of plant extracts as saponins may be a natural method to mitigate methane emissions from ruminants. Diets supplemented with tea saponin included into pelleted concentrates failed to reduce methane emissions in non-lactating and lactating cows. The tendency of this plant extract to improve fiber digestibility in lactating cows needs to be confirmed. Milk production was reduced, most likely because of the tendency for lower intake, but feed efficiency was similar between diets. We suspect that the plant active compound in tea saponin was denatured during the pelleting process.

**Introduction**

Saponins have been considered as promising natural substances for methane ($CH_4$) mitigation in ruminants. This plant extract would have a toxic effect on protozoa through the formation of complex with sterols present in their membrane, inducing cell lysis (Goel and Makkar, 2012). However, the *in vivo* effect of saponins on methanogenesis and protozoa in the rumen presents contradictory results according to the source and supplemented dose. The decrease of protozoa (between 58 and 88%) with saponins supplementation either involved a reduction (-13% with 1% sarsaponin from *Yucca schidigeria*, YS; Lila et al., 2005), an increase (+14% with 4% saponin from *Medicago sativa*; Klita et al., 1996), or no variation (up to 0.13% saponins from YS and *Quillaja saponaria*, QS; Pen et al., 2007; Holtshausen et al., 2009) in $CH_4$ emissions.

Recent reviews highlighted a high anti-methanogenic potential for tea saponin (Wang et al., 2012; Gerber et al., 2013). This novel saponin is extracted from the seeds, leaves and roots of the tea tree from Japan (*Camellia sinensis*) or Sri Lanka (*Camellia assamica*). Reduction of $CH_4$ emissions (g/kg dry matter intake, DMI) with tea saponin (0.25 to 0.5% of DM) supplemented to sheep (Yuan et al., 2007; Mao et al., 2010; Zhou et al., 2011) or steers (Li and Powers, 2012) averaged 26% per percentage added tea saponin. Mao et al. (2010) related this $CH_4$-mitigating effect with a significant reduction of ruminal protozoa concentration (-41%). In addition, the association of dietary strategies acting on both protozoa (linseed, saponin) and methanogens (nitrate) additively lowered methanogenesis *in vitro* (saponin from QS plus nitrate; Patra and Zhongtang, 2013), and *in vivo* (linseed plus nitrate fed to cows; Guyader et al., 2014b). Tea saponin would also improve *in vitro* organic matter (OM) digestibility (+21%; Wei et al., 2012) but this effect has never been tested *in vivo*.

The objective of this study was to test the effect of tea saponin alone or in association with nitrate fed to lactating and non-lactating cows on $CH_4$ emissions, diet digestibility, fermentation parameters and protozoa concentration in the rumen.

**Material and methods**

Two experiments were conducted at the animal facilities of the Experimental Unit UERT at the INRA's Theix Research Centre (Saint-Genès-Champanelle, France). Trial 1 was led from January to June 2013 and trial 2 was led from January to April 2014. Procedures involving animals were performed in accordance with the French Ministry of Agriculture guidelines for animal research and with the applicable EU guidelines and regulations on experiments with animals (http://www2.vet-lyon.fr/ens/expa/acc_regl.html).

*Experimental design and animal feeding in trial 1*

Four multiparous non-lactating Holstein cows fitted with rumen cannulas (initial average BW of 658 ± 26 kg, mean ± s.d.) were randomly assigned to four dietary treatments in a 2 × 2 factorial design, using either calcium nitrate or tea saponin at two different doses (0 and 3% for calcium nitrate; 0 and 0.5% for tea saponin). Each experimental period lasted five weeks, with measures performed in the final two weeks (weeks 4 & 5). All along the experiment, animals were housed in individual stalls. On a dry matter (DM) basis, diets were: 1) control (CON-1), 2) CON-1 with 0.5 % tea saponin (TEA-1), 3) CON-1 with 2.3% nitrate (NIT-1), 4) CON-1 with 0.5% tea saponin and 2.3% nitrate (TEA+NIT-1). The doses of tea saponin and nitrate were calculated to achieve a theoretical CH4 reduction of 20% (Mao et al., 2010; Van Zijderveld et al., 2011) and 40% when distributed alone or in association, respectively.

Diet CON-1 consisted of 50% natural grass hay and 50% concentrate (DM basis; Table 1) and met the maintenance requirements of non-lactating cows (INRA, 2010). Diets were formulated to get similar levels of starch (26.0%), protein (12.2%), NDF (40.1%) and calcium (Ca, 0.67%). Diets were adjusted to have the same nitrogen (N) and Ca concentrations by including urea and calcium carbonate in CON-1 and TEA-1. Forage was distributed without further processing and all other ingredients including tea saponin or nitrate or both were pelleted in concentrates (InVivo NSA, Chierry, France).

Two weeks before starting the experiment, cows were fed CON-1 *ad libitum*. Then, all along the trial, feed was restricted to 90% of individual voluntary feed intakes to ensure complete consumption of the diet. At the beginning of each experimental period, TEA-1, NIT-1 and TEA+NIT-1 concentrates were progressively supplied by replacing CON-1 concentrate. The

TEA-1 concentrate was distributed at maximal dose after a 5-day transition period, whereas the NIT-1 and TEA+NIT-1 concentrates were distributed at their maximal dose after a 10-day transition period.

Feeds were offered twice daily (66% at 0800 h and 34% at 1600 h for hay; 60% between 0800 and 0930 h in three equal portions and 40% between 1600 and 1630 h in two equal portions for concentrates). Forage-to-concentrate ratio was kept as close as possible to the target ratio by adjusting the amounts of feed offered daily based on the composition of the refusals of the previous day. Cows had free access to water throughout the experiment.

**Table 1** Ingredients and chemical composition of the experimental diets (trial 1)

| | Diet[1] | | | |
|---|---|---|---|---|
| | CON-1 | NIT-1 | TEA-1 | TEA+NIT-1 |
| Ingredients (% of DM) | | | | |
| Hay | 50.00 | 50.00 | 50.00 | 50.00 |
| Pelleted concentrates | | | | |
| Wheat | 25.23 | 25.23 | 25.23 | 25.23 |
| Maize | 15.00 | 15.00 | 15.00 | 15.00 |
| Calcium nitrate[2] | 0 | 3.00 | 0 | 3.00 |
| Tea saponin extract[3] | 0 | 0 | 0.77 | 0.77 |
| Calcium carbonate | 1.70 | 0 | 1.70 | 0 |
| Urea | 1.22 | 0 | 1.22 | 0 |
| Dehydrated beet pulp | 4.08 | 4.00 | 3.31 | 3.23 |
| Molasses beet | 1.00 | 1.00 | 1.00 | 1.00 |
| Binder | 1.00 | 1.00 | 1.00 | 1.00 |
| Mineral-vitamin mix | 0.75 | 0.75 | 0.75 | 0.75 |
| Aroma | 0.02 | 0.02 | 0.02 | 0.02 |
| Chemical composition (% of DM) | | | | |
| OM | 91.3 | 91.5 | 91.4 | 91.4 |
| CP | 12.7 | 12.2 | 12.4 | 11.6 |
| NDF | 40.1 | 40.2 | 40.2 | 40.0 |
| ADF | 23.3 | 23.1 | 23.1 | 23.1 |
| Starch | 25.4 | 25.7 | 26.3 | 26.4 |
| GE (MJ/kg of DM) | 17.4 | 16.6 | 17.5 | 16.5 |

[1] CON-1 = diet control; NIT-1 = diet CON-1 containing 2.3% nitrate; TEA-1 = diet CON-1 containing 0.5% tea saponin; TEA+NIT-1 = diet CON-1 containing 0.5% tea saponin and 2.3% nitrate.

[2] $5Ca(NO_3)_2.NH_4NO_3.10H_2O$; 75% $NO_3$ in DM (Phytosem, Pont-du-Château, France).

[3] 688 g saponins/kg of DM according to supplier (Choisun Tea Sci-Tech Co. Ltd., Hangzhou, Zhejiang, China) indications.

*Experimental design and animal feeding in trial 2*

Eight lactating Holstein cows (four primiparous and four multiparous) were used. At the beginning of the experiment, the average BW was 629 ± 53 kg, milk production was 29 ± 7

kg and number of days in milk was 106 ± 21 days. Cows were separated into two groups balanced for number of primiparous, calving date, and milk production. The two groups were conducted in a 2 × 2 crossover design. Each experimental period lasted five weeks with the two last weeks for measurement (weeks 4 & 5). Cows were housed in a freestall barn except during the measurement weeks in which they were tied individually.

**Table 2** Ingredients and chemical composition of the experimental diets (trial 2)

| | Diet[1] | |
|---|---|---|
| | CON-2 | TEA-2 |
| Ingredients (% of DM) | | |
| Maize silage | 54.00 | 54.00 |
| Hay | 6.00 | 6.00 |
| Pelleted concentrates | | |
| Maize | 11.88 | 11.88 |
| Barley | 3.36 | 2.96 |
| Soybean meal | 5.24 | 5.24 |
| Rapeseed meal | 2.00 | 2.00 |
| Soybean hulls | 6.60 | 6.60 |
| Wheat bran | 6.00 | 5.24 |
| Dehydrated beet pulp | 0.94 | 0.94 |
| Urea | 0.80 | 0.80 |
| Calcium carbonate | 1.13 | 1.13 |
| Dicalcium phosphate | 0.44 | 0.44 |
| Molasses beet | 1.20 | 1.60 |
| Mineral-vitamin mix | 0.20 | 0.20 |
| Salt | 0.17 | 0.17 |
| Fungicide | 0.02 | 0.02 |
| Aroma | 0.02 | 0.02 |
| Tea saponin extract[2] | 0.00 | 0.76 |
| Chemical composition (% of DM) | | |
| OM | 93.0 | 93.1 |
| CP | 16.1 | 16.1 |
| NDF | 35.1 | 35.6 |
| ADF | 18.4 | 18.7 |
| Starch | 28.2 | 27.8 |
| GE (MJ/kg of DM) | 17.7 | 17.9 |

[1] CON-2= diet control; TEA-2 = diet CON-2 containing 0.5% tea saponin.

[2] 689 g saponins/kg of DM according to supplier (Choisun Tea Sci-Tech Co. Ltd., Hangzhou, Zhejiang, China) indications.

Each group of cows received two dietary treatments consisting in (on a DM basis): 1) control (CON-2), 2) CON-2 with 0.5% tea saponin (TEA-2). Dosage of tea saponin and manufacturer were similar to trial 1 but the extract came from different batches as purchased separately. Diet CON-2 was made of 54% maize silage, 6% hay and 40% pelleted concentrates (InVivo

NSA, Longué-Jumelles, France; Table 2) and met the requirements of lactating dairy cows (INRA, 2010). Diets were equivalent in terms of starch (28.0%), crude protein (16.1%) and fiber (35.4%).

Two weeks before starting the experiment, cows were fed *ad libitum* with CON-2. Then, all along the experiment, cows were fed ad libitum, except during measurement weeks in which offered feed was restricted to 95% of individual voluntary feed intake. At the beginning of each experimental period, TEA-2 concentrate was progressively supplied by replacing CON-2 concentrate, to achieve the maximal dose after a one week transition period. During the experiment, hay was offered once daily (0800 h) and maize silage mixed with concentrates was distributed two times per day (66% at 0930 h and 34% at 1600 h). Forage-to-concentrate ratio was kept as close as possible to the target ratio by adjusting the amounts of feed offered weekly based on the composition of the refusals of the previous week. Cows had free access to water throughout the experiment.

*Measurements and analyses for trials 1 & 2*

*Intake.* During the 2 trials, offered feed and refusals were weighed and recorded daily to estimate DMI. Feed (hay and concentrate for trial 1; silage, hay and concentrate for trial 2) were sampled as described previously (Guyader et al., 2014b). Briefly, one sample of each feed was taken on two days during weeks 4 and 5. For each sample, one aliquote was used to determine DM (103°C for 24h) and another aliquote was stored at 4°C (hay and concentrate) or -20°C before freeze drying (maize silage). Refusals DM content was determined if they exceeded 1 kg/day and per animal in weeks 4 and 5. At the end of the experiment, each feed samples were pooled per treatment and ground (1 mm screen) before chemical analyses (InVivo Labs, Saint-Nolff, France for trial 1; InVivo Labs, Chierry, France for trial 2).

Organic matter was determined by ashing at 550°C for 6h (method 942.05; AOAC, 2005). Fiber (NDF and ADF) was determined by sequential procedures (Van Soest et al., 1991) after pretreatment with amylase, and expressed exclusive of residual ash. Total N was analyzed by combustion according to the Dumas method (method 968.06; AOAC, 2005), and CP content was calculated as N × 6.25. Starch was analyzed using an enzymatic method (Faisant et al., 1995) and gross energy (GE) was analyzed by isoperibolic calorimetry (C200 model, IKA, Staufen, Germany).

*Liveweight and blood methemoglobin.* Animals were weighed at the end of each experimental period. In trial 1, levels of blood methemoglobin (metHb) were controlled 3h after morning meal for animals fed nitrate (NIT-1 and TEA+NIT-1). One control sample was taken from all animals the week preceding the start of the experiment. Then, blood was sampled at days 3, 5, 10, 12, 17, 19 and 29 of each experimental period. Blood was sampled from jugular vein and packed onto ice before metHb content analysis (Kaplan, 1965) within 1h at the nearest hospital (CHU Gabriel Montpied, Clermont-Ferrand, France).

*Methane emissions.* Kinetics of $CH_4$ emissions were determined using open chambers in week 4 as described in Guyader et al. (2014b), during four (trial 1) and two (trial 2) consecutive days. Chambers rear doors were opened twice daily for cleaning and milking, and front doors were opened for each feed distribution (five times per day for trial 1 and three times per day for trial 2). In total, doors were opened on average 15 min/day (trial 1) and 30 min/day (trial 2). As far as possible, doors were not opened during gases concentration analysis or deleted if it was the case. Air fluxes were not corrected for environmental data, as trial 1 showed that this correction did not influence final values.

*Digestibility and nitrogen balance.* Total tract digestibility of nutrients and N balance were determined via daily total and separate collection of feces and urine in week 5. Collection lasted six days in trial 1 and five days in trial 2. Each day, after weighing and mixing of feces, one aliquote (1%) was used to determine DM (103°C for 24 h) and another aliquote (1%) was pooled per week and per animal before freezing (-20°C). At the end of trials, samples were defrosted and dried (trial 1) or freeze-dried (trial 2) before grinding (1 mm screen). Chemical composition (OM, NDF, ADF, CP) was analyzed similarly to feed.

Urine was collected in vessels containing 500 mL sulfuric acid 3 M to maintain a urine pH lower than 3 to avoid N volatilization. Each day, after weighing, one aliquote (1%) was pooled per week and per animal before freezing (-20°C). At the end of each trial, samples were defrosted and N content was determined according to Kjeldahl method (InVivo Labs, Chierry, France; method 2001.11; AOAC, 2005) as it was not possible to apply the Dumas method on fresh urine. In trial 1, a second aliquote (0.25%) was diluted (1:4) with distilled water and pooled per week and per animal before freezing (-20°C). At the end of the trial, samples were defrosted and concentration in derivatives of puric bases (DPB; xanthin, hypoxanthin, allantoïne, uric acid) was determined by high pressure liquid chromatography (Shingfield and Offer, 1999) to assess microbial synthesis within the rumen.

*Rumen fermentation parameters.* Rumen content was sampled in the ventral sac of each cow through the cannula, 3h after morning meal, during two non-consecutive days in week 5 (trial 1) or by stomach tubing, 3.5h after the morning meal, on the last day of week 5 (trial 2). All rumen samples were strained through a polyester monofilament fabric (250 µm pore size) and filtrate was subsampled for volatile fatty acids (VFA, 0.8 mL filtrate in 0.5 mL of a 0.5 M HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid), ammonia ($NH_3$, 1 mL filtrate in 0.1 mL of 5% orthophosphoric acid), and protozoa (2 mL filtrate in 2 mL of methyl green-formalin solution) concentrations analyses. In trial 1, lactate (3 mL filtrate without preservative), nitrate and nitrite (20 mL filtrate without preservative) concentrations were also determined as well as dynamics of rumen pH which was followed during six consecutive days in week 4 with boluses (eBolus, eCow, Exeter, United Kingdom; Guyader et al., 2014b).

Samples were stored at -20°C before analysis, except for protozoa samples which were stored at room temperature and away from direct light until counting. Concentrations of VFA and $NH_3$ were analyzed by gas chromatography with a flame ionization detector and by colorimetry, respectively (Morgavi et al., 2008). Lactate concentrations were determined by colorimetry (D/L-lactic acid, BioSentec, Auzeville-Tolosane, France). Nitrate and nitrite concentrations were analyzed by spectrometry (Laboratoire Vétérinaire et Biologique, Lempdes, France). Protozoa were counted by microscopy and categorized as either small (<100 µm) or large (>100 µm) entodiniomorphs, or as holotrichs (Dasytricha or Isotricha) (Williams and Coleman, 1992). Protozoa concentrations were $\log_{10}$-transformed before statistical analysis.

*Milk yield and composition in trial 2.* Milk production was daily quantified. Milk composition was determined at each milking on samples (30 mL) taken one day in week 4, mixed with potassium bichromate (Merck, Fontenay Sous Bois, France), and stored at 4°C. Milk fat, protein and lactose content were analyzed by infrared spectrometry with a 3-channel spectrophotometer (Galilait, Theix, France; method 972.16; AOAC, 1990) and urea concentration was determined by colorimetry (Galilait, Theix, France; Potts, 1967).

*Statistical analyses*

All statistical analyses were carried out with the mixed procedure of SAS (Version 9.2; SAS Institute, 2009). As sampling day effect (n = 2 for rumen fermentation parameters in trial 1; n

= 4 or 2 for $CH_4$ emissions in trials 1 and 2, respectively) was never significant, this factor was not considered in subsequent analyses, and all data were averaged per period.

In trial 1, the statistical model included the random effect of cow (n = 4) and fixed effects of period (n = 4), nitrate (CON-1 and TEA-1 versus NIT-1 and TEA+NIT-1), tea saponin (CON-1 and NIT-1 versus TEA-1 and TEA+NIT-1) and the interaction nitrate × tea saponin. In trial 2, one animal passed away during the second period after a fall not linked with the trial. All data for this animal were deleted in further statistical analyses. The statistical model included the animal (n = 7) as random effect, and period (n = 2) and diet (n = 2) as fixed effects.

Daily kinetics of ruminal pH (trial 1) and $CH_4$ emissions (trials 1 and 2) were analyzed by repeated time. Hour (n = 24 for both trials) was treated as a repeated measurement with compound symmetry as covariance structure. In trial 1, the model included the fixed effects of period, hour, nitrate, tea saponin, nitrate × tea saponin and the interactions between hour and dietary treatments (tea saponin × hour, nitrate × hour, tea saponin × nitrate × hour). In trial 2, the model included period, diet, hour and diet × hour interactions as fixed effects.

Differences between diets were considered significant at $P<0.05$, and trends were discussed at $0.05\leq P\leq 0.1$. Least squares means are reported throughout.

## Results

### *Trial 1 on non-lactating cows*

*Animals weight and metHb levels.* At the end of the trial, animals weighed 699 ± 42 kg, which corresponded to an average weight gain of 10 kg per animal and per period. Levels of blood metHb progressively increased until the end of the second week of adaptation, before going down and remaining at stable and low levels at the beginning of measurement weeks (Supplementary material Figure S1). The maximal level reached by one animal fed NIT-1 was 25.9% on day 12.

*Intake, diet digestibility and nitrogen balance (Table 3).* Both nitrate and tea saponin reduced daily intake (DM, OM, NDF, ADF and GE; $P<0.05$), with an additive effect between tea saponin and nitrate (tea saponin × nitrate, $P>0.05$). Nutrient digestibility was similar between diets ($P>0.05$), with an average DM digestibility of 63.7%. Saponin-containing diets (TEA-1 and TEA+NIT-1) tended to improve NDF digestibility ($P=0.126$) and nitrate-containing diets

(NIT-1 and TEA+NIT-1) tended to increase ADF digestibility (*P*=0.073). Nitrogen balance was positive (+17.3 g/day) and similar between diets.

**Table 3** Daily nutrient intake, total tract digestibility and N balance of non-lactating cows fed diets containing tea saponin and calcium nitrate alone or in association (n = 4; trial 1)

| | Diet[1] | | | | | *P*-value[2] | | |
|---|---|---|---|---|---|---|---|---|
| | CON-1 | NIT-1 | TEA-1 | TEA+NIT-1 | SEM | Saponin | Nitrate | Saponin × nitrate |
| Daily nutrient intake | | | | | | | | |
| DM (kg/day) | 12.3 | 12.0 | 12.0 | 11.8 | 0.40 | 0.032 | 0.040 | 0.914 |
| OM (kg/day) | 11.2 | 11.0 | 11.0 | 10.7 | 0.37 | 0.032 | 0.048 | 0.756 |
| NDF (kg/day) | 4.93 | 4.84 | 4.83 | 4.71 | 0.164 | 0.032 | 0.047 | 0.758 |
| ADF (kg/day) | 2.86 | 2.79 | 2.77 | 2.71 | 0.095 | 0.018 | 0.044 | 0.962 |
| GE (MJ/day) | 214 | 200 | 210 | 194 | 6.9 | 0.021 | <0.001 | 0.548 |
| Total tract digestibility (%) | | | | | | | | |
| DM | 62.8 | 63.8 | 64.5 | 63.7 | 1.15 | 0.270 | 0.845 | 0.220 |
| OM | 66.9 | 67.9 | 68.1 | 67.7 | 1.11 | 0.336 | 0.451 | 0.180 |
| NDF | 42.3 | 43.7 | 45.2 | 44.6 | 2.60 | 0.126 | 0.697 | 0.369 |
| ADF | 41.9 | 44.8 | 44.3 | 45.3 | 2.83 | 0.160 | 0.073 | 0.331 |
| CP | 59.1 | 54.4 | 58.6 | 55.1 | 3.35 | 0.972 | 0.241 | 0.852 |
| N balance (g/day) | | | | | | | | |
| N intake | 247.5 | 232.5 | 242.5 | 217.5 | 8.54 | 0.003 | <0.001 | 0.050 |
| N in feces | 102.1 | 108.4 | 99.2 | 97.9 | 10.22 | 0.458 | 0.778 | 0.671 |
| N in urine | 123.1 | 106.6 | 133.4 | 103.2 | 9.47 | 0.638 | 0.016 | 0.363 |
| N in feces + urine | 225.2 | 215.0 | 232.6 | 201.1 | 14.56 | 0.763 | 0.089 | 0.339 |
| N balance | 25.0 | 20.4 | 6.8 | 16.9 | 10.79 | 0.286 | 0.778 | 0.459 |

[1] CON-1 = diet control; NIT-1 = diet CON-1 containing 2.3% nitrate; TEA-1 = diet CON-1 containing 0.5% tea saponin; TEA+NIT-1 = diet CON-1 containing 0.5% tea saponin and 2.3% nitrate.

[2] Saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); Nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

*Methane emissions (Table 4).* Animals fed TEA-1 produced the same quantities of $CH_4$ (expressed as g/day, g/kg DMI, g/kg digested DM, g/kg digested OM, g/kg digested NDF, % of GE intake) than animals fed CON-1. Animals fed nitrate-containing diets (NIT-1 and TEA+NIT-1) produced the same quantities of $CH_4$ but in a lower amount than CON-1 (-28% on average; $P<0.05$). Kinetics of $CH_4$ emissions (Supplementary material Figure S2) confirmed the absence of $CH_4$-mitigating effect of tea saponin fed alone all along the day. Inversely, nitrate-containing diets induced lower emissions during 3h following meals before rising to similar levels than CON-1.

**Table 4** Methane emissions of non-lactating cows fed diets containing tea saponin and calcium nitrate alone or in association (n = 4; trial 1)

| | Diet[1] | | | | | *P*-value[2] | | |
|---|---|---|---|---|---|---|---|---|
| | CON-1 | NIT-1 | TEA-1 | TEA+NIT-1 | SEM | Saponin | Nitrate | Saponin × nitrate |
| g $CH_4$/day | 312.3 | 219.2 | 294.0 | 206.3 | 13.37 | 0.248 | <0.001 | 0.830 |
| g $CH_4$/kg DMI | 25.4 | 18.6 | 24.6 | 17.8 | 1.41 | 0.529 | 0.001 | 0.973 |
| g $CH_4$/kg dDM | 40.5 | 29.1 | 38.3 | 28.1 | 2.30 | 0.446 | 0.002 | 0.768 |
| g $CH_4$/kg dOM | 37.9 | 27.4 | 36.3 | 26.4 | 2.11 | 0.488 | 0.001 | 0.846 |
| g $CH_4$/kg dNDF | 60.2 | 42.5 | 55.2 | 41.4 | 4.29 | 0.395 | 0.003 | 0.571 |
| % of GE intake | 7.3 | 5.6 | 7.0 | 5.4 | 0.42 | 0.519 | 0.003 | 0.956 |

[1] CON-1 = diet control; NIT-1 = diet CON-1 containing 2.3% nitrate; TEA-1 = diet CON-1 containing 0.5% tea saponin; TEA+NIT-1 = diet CON-1 containing 0.5% tea saponin and 2.3% nitrate.

[2] Saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); Nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

*Rumen fermentation parameters and protozoa concentrations (Table 5).* Tea saponin fed alone increased total VFA concentrations after feeding compared to CON-1 (+19%; $P<0.05$) without modifying VFA profile. Diets supplemented with nitrate (NIT-1 and TEA+NIT-1) increased acetate proportion (+10% on average; $P<0.01$), reduced butyrate proportion (-39% on average; $P<0.01$) and reduced ammonia concentrations (-23.6% on average; $P<0.05$). No treatment affected nitrite concentrations and nitrate was never detected in the rumen. Average daily pH was similar between diets (6.20 on average), despite a reduction for TEA-1 between 3 and 5h after the morning meal and between 1 and 4h after the afternoon meal (Supplementary material Figure S3). Saponin-containing diets (TEA-1 and TEA+NIT-1) tended to increase protozoa concentration ($P<0.10$).

**Table 5** Daily average pH, rumen fermentation characteristics and protozoa concentration 3 h after feeding non-lactating cows with diets containing tea saponin and calcium nitrate alone or in association (n = 4; trial 1)

| | Diet[1] | | | | | *P*-value[2] | | |
|---|---|---|---|---|---|---|---|---|
| | CON-1 | NIT-1 | TEA-1 | TEA+NIT-1 | SEM | Saponin | Nitrate | Saponin × nitrate |
| Total VFA (m*M*) | 101.50 | 98.43 | 120.58 | 98.13 | 5.515 | 0.013 | 0.003 | 0.011 |
| VFA profile (%) | | | | | | | | |
| Acetate (C2) | 67.83 | 75.53 | 69.10 | 73.56 | 1.512 | 0.787 | 0.003 | 0.234 |
| Propionate (C3) | 16.57 | 14.68 | 16.39 | 16.38 | 1.911 | 0.489 | 0.397 | 0.401 |
| Butyrate (C4) | 11.64 | 7.01 | 11.08 | 7.16 | 0.854 | 0.813 | 0.002 | 0.678 |
| Minor VFA[3] | 3.97 | 2.78 | 3.43 | 2.93 | 0.382 | 0.563 | 0.040 | 0.322 |
| C2/C3 | 4.27 | 5.33 | 4.46 | 4.65 | 0.554 | 0.507 | 0.120 | 0.260 |
| (C2+C4)/C3 | 5.00 | 5.82 | 5.18 | 5.11 | 0.628 | 0.501 | 0.347 | 0.279 |
| $NH_3$-N (m*M*) | 18.32 | 14.84 | 18.42 | 13.15 | 1.790 | 0.570 | 0.016 | 0.525 |
| Total lactate (m*M*) | 0.80 | 0.80 | 0.93 | 0.51 | 0.196 | 0.624 | 0.214 | 0.210 |
| Nitrite (mg/L) | 0.24 | 4.17 | 0.24 | 1.63 | 1.756 | 0.482 | 0.172 | 0.482 |
| pH[4] | 6.24 | 6.31 | 6.01 | 6.22 | 0.104 | 0.137 | 0.187 | 0.480 |
| Total protozoa ($log_{10}$/mL) | 5.38 | 5.40 | 5.53 | 5.58 | 0.146 | 0.067 | 0.655 | 0.875 |

[1] CON-1 = diet control; NIT-1 = diet CON-1 containing 2.3% nitrate; TEA-1 = diet CON-1 containing 0.5% tea saponin; TEA+NIT-1 = diet CON-1 containing 0.5% tea saponin and 2.3% nitrate.

[2] Saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); Nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

[3] Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.

[4] Daily average.

*Trial 2 on lactating cows*

Animals lost 11 kg on average per period, to end with a final BW of 608 ± 33 kg.

*Intake, diet digestibility and nitrogen balance (Table 6).* Diet TEA-2 numerically reduced daily DMI (-2.3 kg/day), and did not affect intake of OM, NDF, ADF and GE. Nutrients digestibility (DM, OM, NDF, CP) was similar between diets with an average DM digestibility of 66.2%, but TEA-2 tended to improve ADF digestibility (+8%; $P<0.10$). N balance was positive and similar between CON-2 and TEA-2 (+54.6 g/day on average).

**Table 6** Daily nutrient intake, total tract digestibility and N balance of lactating cows fed a diet containing tea saponin (n = 7; trial 2)

| | Diet[1] | | | |
|---|---|---|---|---|
| | CON-2 | TEA-2 | SEM | *P*-value |
| Daily nutrient intake | | | | |
| DM (kg/day) | 20.0 | 17.7 | 1.23 | 0.109 |
| OM (kg/day) | 18.6 | 16.5 | 1.15 | 0.111 |
| NDF (kg/day) | 7.04 | 6.31 | 0.434 | 0.143 |
| ADF (kg/day) | 3.69 | 3.31 | 0.227 | 0.139 |
| GE (MJ/day) | 354 | 316 | 21.8 | 0.129 |
| Total tract digestibility (%) | | | | |
| DM | 65.8 | 66.6 | 0.78 | 0.362 |
| OM | 67.5 | 68.4 | 0.77 | 0.359 |
| NDF | 48.3 | 52.1 | 1.55 | 0.147 |
| ADF | 43.9 | 47.9 | 1.38 | 0.086 |
| CP | 63.9 | 63.0 | 0.99 | 0.345 |
| N balance (g/day) | | | | |
| N intake | 515.6 | 457.4 | 33.02 | 0.118 |
| N in feces | 186.4 | 169.2 | 14.07 | 0.254 |
| N in urine | 136.3 | 120.6 | 5.75 | 0.112 |
| N in feces + urine | 322.8 | 289.8 | 18.36 | 0.199 |
| N in milk | 143.9 | 123.0 | 14.54 | 0.486 |
| N balance | 52.2 | 56.9 | 12.74 | 0.878 |

[1] CON-2 = diet control; TEA-2 = diet CON-2 containing 0.5% tea saponin.

*Milk production and methane emissions (Table 7).* Diet TEA-2 reduced milk production by 18% (23.6 *versus* 28.9 kg/day; P<0.001) without affecting milk content in fat (34.3 g/kg on average), protein (30.8 g/kg on average), lactose (50.5 g/kg on average) and urea (20.0 mg/dL on average). Feed efficiency was similar between CON-2 and TEA-2 (1.39 kg milk/kg DMI on average).

Expressed in g/day, $CH_4$ emissions were similar between CON-2 and TEA-2, and were higher for TEA-2 when expressed in g/kg DMI (+12.7%; *P*<0.001), g/kg milk (+20.9%; *P*<0.05), g/kg digested nutrients (+11.9% for OM; *P*<0.05) or as a percentage of GE intake (+12.8%; *P*<0.001). These differences between diets were maintained all along the day as observed on daily kinetics of $CH_4$ emissions (Supplementary material Figure S4).

**Table 7** Milk production and $CH_4$ emission of lactating cows fed a diet containing tea saponin (n = 7; trial 2)

| | Diet[1] | | | |
|---|---|---|---|---|
| | CON-2 | TEA-2 | SEM | *P*-value |
| Milk yield (kg/day) | 28.9 | 23.6 | 1.97 | <0.001 |
| Feed efficiency[2] (kg milk/kg DMI) | 1.45 | 1.33 | 0.083 | 0.251 |
| Fat concentration (g/kg) | 30.3 | 38.2 | 3.48 | 0.321 |
| Protein concentration (g/kg) | 31.6 | 29.9 | 0.70 | 0.326 |
| Lactose concentration (g/kg) | 50.2 | 50.7 | 0.92 | 0.052 |
| Urea concentration (mg/dL) | 21.7 | 18.2 | 4.36 | 0.611 |
| $CH_4$ emissions | | | | |
| g $CH_4$/day | 435.2 | 442.2 | 38.69 | 0.840 |
| g $CH_4$/kg DMI | 21.3 | 24.7 | 1.10 | 0.004 |
| g $CH_4$/kg milk | 15.1 | 19.1 | 1.22 | 0.018 |
| g $CH_4$/kg dDM | 32.5 | 37.0 | 1.61 | 0.021 |
| g $CH_4$/kg dOM | 34.1 | 38.7 | 1.69 | 0.023 |
| g $CH_4$/kg dNDF | 126.2 | 133.2 | 5.99 | 0.454 |
| % of GE intake | 6.01 | 6.89 | 0.310 | 0.006 |

[1] CON-2 = diet control; TEA-2 = diet CON-2 containing 0.5% tea saponin.
[2] Feed efficiency = milk yield/DMI.

*Rumen fermentation parameters and protozoa concentrations (Table 8).* Concentrations in $NH_3$ and total VFA were similar between CON-2 and TEA-2 (15.1 and 105.2 mM, respectively). The VFA profile differed only in acetate proportion, which was higher for TEA-2 (+6.2%; $P<0.05$) inducing a tendency for a higher C2/C3 ratio compared to CON-2 ($P<0.10$). Protozoa concentrations were similar between diets (5.1 $\log_{10}$/mL on average).

**Table 8** Rumen fermentation characteristics and protozoa concentration 3 h after feeding lactating cows with a diet containing tea saponin (n = 7; trial 2)

| | Diet[1] | | | |
|---|---|---|---|---|
| | CON-2 | TEA-2 | SEM | *P*-value |
| $NH_3$-N (m*M*) | 16.08 | 14.15 | 2.763 | 0.643 |
| Total VFA (m*M*) | 107.07 | 103.32 | 10.720 | 0.806 |
| VFA profile (%) | | | | |
| Acetate (C2) | 55.68 | 61.87 | 2.005 | 0.035 |
| Propionate (C3) | 23.25 | 20.47 | 1.650 | 0.185 |
| Butyrate (C4) | 16.57 | 13.72 | 1.429 | 0.199 |
| Minor VFA[2] | 4.26 | 3.95 | 0.326 | 0.516 |
| C2/C3 | 2.49 | 3.07 | 0.226 | 0.062 |
| (C2+C4)/C3 | 3.26 | 3.75 | 0.272 | 0.176 |
| Total protozoa ($\log_{10}$/mL) | 5.02 | 5.18 | 0.117 | 0.360 |

[1] CON-2 = diet control; TEA-2 = diet CON-2 containing 0.5% tea saponin.
[2] Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.

**Discussion**

*Intake and reduction of lactating performances in cows fed tea saponin*

In both trials, intake was reduced by tea saponin supplementation, even if the plant extract was included into pelleted concentrates which should have improved its palatability thanks to the presence of aroma. We also faced difficulties to feed tea saponin as a powder, as handling of the powder led to respiratory irritation problems for users and animals refused to eat it. This issue has never been highlighted in previous studies testing this plant extract (Mao et al., 2010; Zhou et al., 2011; Li and Powers, 2012).

Tea saponin clearly reduced milk production without affecting milk composition. The reduction of milk yield can be explained by the tendency for a lower DMI, as feed efficiency was similar between diets. To our knowledge, the negative effect of tea saponin on lactating performances of dairy cattle has never been observed. Instead, inconsistent results have been reported on beef cattle and lambs. Mao et al. (2010) did not observe differences in growth of lambs supplemented with 0.5% tea saponin. With steers, Li and Powers (2012) reported no effect of 0.05% tea saponin on the average daily weight gain, whereas a higher dose (0.11%) reduced the average daily weight gain by 80% linked to a drop of DMI (-27%). Overall results show that a dose response study on dairy cattle is required to complete this work.

*Absence of positive methane mitigating effect of tea saponin*

Tea saponin supplementation (0.5% DM) did not affect $CH_4$ emissions (g/kg DMI) of non-lactating cows and increased $CH_4$ emissions (g/kg DMI) of lactating cows, after 4 weeks of feeding saponin. This result is linked to the absence of the expected reduction of ruminal protozoa in both studies suggesting an adaptation of this population. Indeed, in sheep, a decrease of protozoa number after 4 days of feeding saponins (*Sesbania sesban*) was reported but this population recovered 10 days later (Newbold et al., 1997). The absence of $CH_4$-mitigating effect of tea saponin was reported previously on steers but animals were fed low tea saponin doses (0.11% maximum; Li and Powers, 2012). However, with similar doses than ours (0.5% tea saponin), $CH_4$ emissions (g/kg DMI) were reduced by 27% (Mao et al., 2010) and 11% (Zhou et al., 2011) in sheep, and were linked to a reduction of protozoa concentrations (-41% and -43% of total bacterial 16S rDNA, respectively) after 3-8 weeks saponin feeding.

Several reasons may explain the inefficiency of our tea saponin extract on methanogenesis and on associated rumen microbial and fermentative parameters. In our trials, tea saponin was

included into granulated concentrates whereas it was distributed as a powder in other studies. During the pelleting process, the saponin was heated (~40°C), which may have damage its anti-methanogenic and -protozoal properties. Indeed, a modification of the miscellaneous structure of QS was already observed after heating between 20 and 60°C (Mitra and Dungan, 1997). An animal species effect (sheep *versus* cattle) may be also considered. Finally, we cannot exclude an effect of the batch production; plant maturity, geographical area of production and extraction methods are three parameters affecting the final concentration and quality of the saponin (Li and Powers, 2012).

The mode of action of nitrate to mitigate methanogenesis is different from saponins as it does not reduce protozoa. Nitrate may not only act as a hydrogen-sink but may also have a direct inhibiting effect towards rumen methanogens (Guyader et al., 2014a). Nitrate fed alone reduced $CH_4$ emissions related to DMI by 27%, corresponding to a 12% reduction per percentage unit of nitrate fed. This result confirms once more time the efficiency and repeatability of the nitrate $CH_4$-mitigating effect in cattle (Hulshof et al., 2012; Guyader et al., 2014b; Veneman et al., 2014). Moreover, a recent meta-analysis reported a linear dose-response effect of nitrate (0.3 to 1.2 g/kg BW/day) on enteric $CH_4$ emissions with a reduction of 12% of $CH_4$ yield (g/kg DMI) per 0.1 g added nitrate/kg BW/day (Lee and Beauchemin, 2014). Association of nitrate plus tea saponin did not accentuate the $CH_4$-mitigating effect of nitrate, suggesting that the $CH_4$ reduction with this association was linked to the nitrate effect.

Nitrate fed alone or in association with tea saponin to non-lactating cows increased acetate without changing propionate concentrations in the rumen, which confirmed previous findings (Nolan et al., 2010; Hulshof et al., 2012; Veneman et al., 2014). Increased acetate concentration may compensate the hydrogen deficiency in the rumen (Janssen, 2010) linked to nitrate reduction.

*Improvement of fiber digestibility with tea saponin*

Tea saponin did not modify diet digestibility of non-lactating cows, whereas with lactating cows, it tended to improve ADF digestibility (+4 units). To our knowledge, our study is the first one to show a beneficial effect of tea saponin on nutrient digestibility of cattle. This effect was not reported on goats supplemented with low doses (0.04, 0.06 and 0.08%; Zhou et al., 2012). Generally, saponins have an undermined effect on diet digestibility, which seems to be linked to their source and dose. Only Pen et al. (2007) observed an increased NDF digestibility (+3.7 units) on ovine supplemented with 0.08% saponin from QS. Most authors reported no effect of saponins on diet digestibility in bovine (0.03% saponin from YS or QS,

Holtshausen et al., 2009) or in ovine (0.13% saponin from YS, Pen et al., 2007; 1-4% saponin from *Medicago sativa*, Klita et al., 1996). A depressive effect on fiber digestibility was even shown with 0.5 and 1% saponin from YS (-2.6 and -2.9 units, respectively; Lila et al., 2005) and with 1% saponin extracted from the tropical tree *Sapindus saponaria* (-3 units, Hess et al., 2004).

Nitrate supplementation did not affect diet digestibility and N balance in both trials, confirming previous studies on sheep (Nolan et al., 2010) and lactating cows (Van Zijderveld et al., 2011; Guyader et al., 2014b) supplemented up to 2.5% nitrate. Nitrate was well metabolized by the animals and can substitute urea as a non-protein N source in diets low in fermentescible N content (Leng, 2008). Moreover, the absence of animals' health issue in terms of methemoglobinemia supports the use of this chemical at the farm scale under controlled conditions. It is recommended to feed animals with maximum doses of 1% nitrate (Doreau et al., 2014) and to apply a long enough adaptation period (Lee and Beauchemin, 2014).

In conclusion, tea saponin supplementation did not reduce $CH_4$ emissions and rumen protozoa concentrations in cattle. The inefficiency may be explained by the denaturation of the active compound of the plant when heating during the pelleting process. To test this hypothesis, an *in vitro* experiment may be carried out to compare gas production and composition and protozoa number with pelleted or non-pelleted tea saponin supplementation. This plant extract tended to increase fiber digestibility of lactating cows, without improving animals' performances. Further work is required to improve tea saponin palatability and to confirm its positive effect on digestibility via a dose response study.

**Acknowledgements**

J. Guyader is recipient of an INRA-Région Auvergne PhD scholarship. The authors thank L. Genestoux for laboratory analysis, L. Mouly, D. Roux, S. Rudel for animal care, the Observatoire de Physique du Globe in Clermont-Ferrand for providing weather data.

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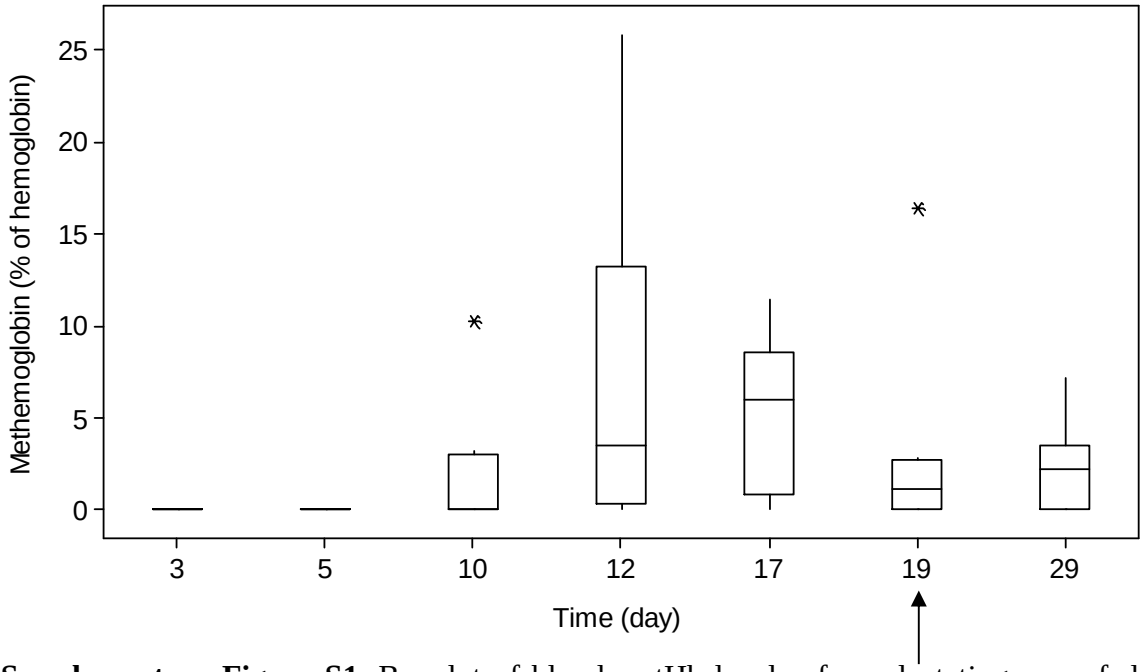
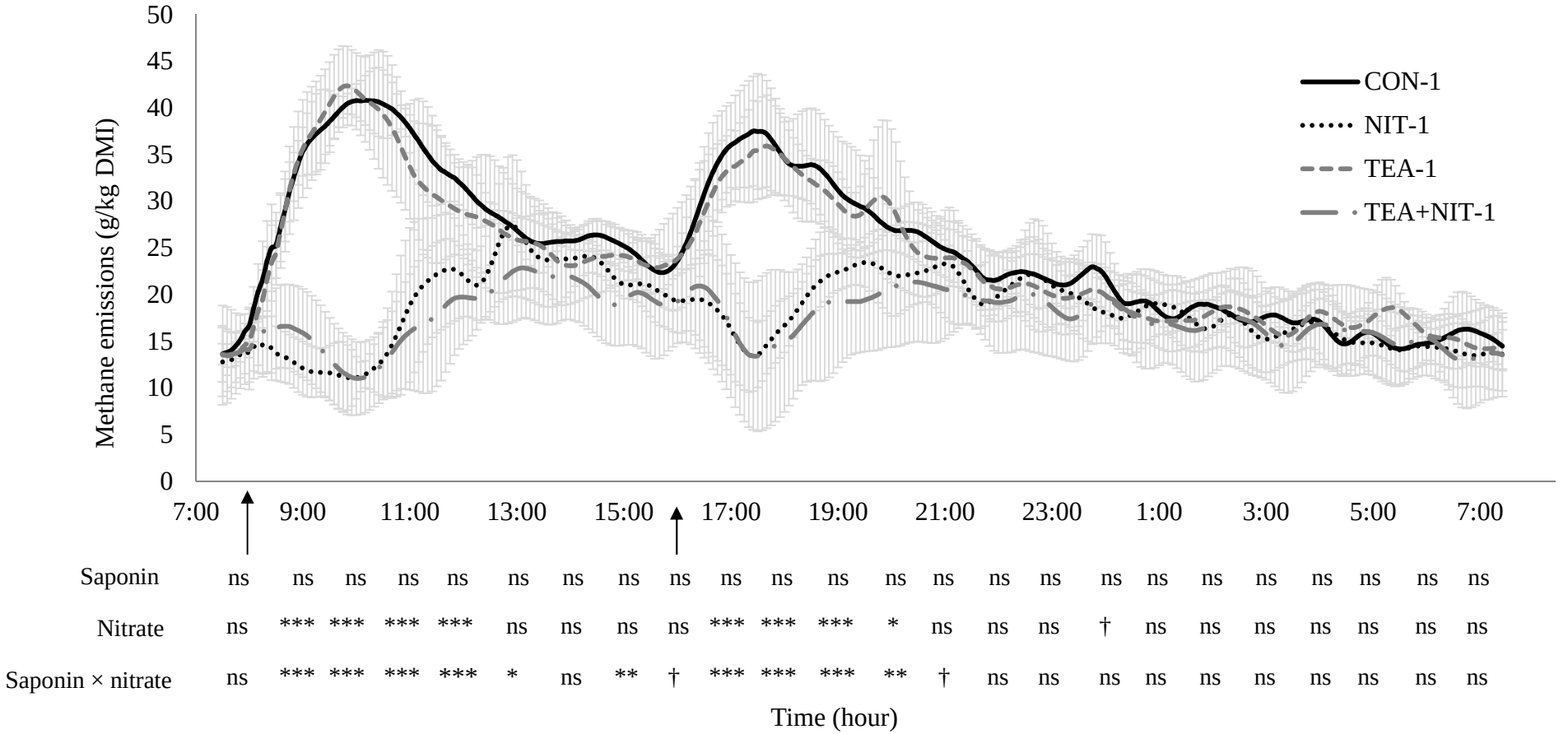
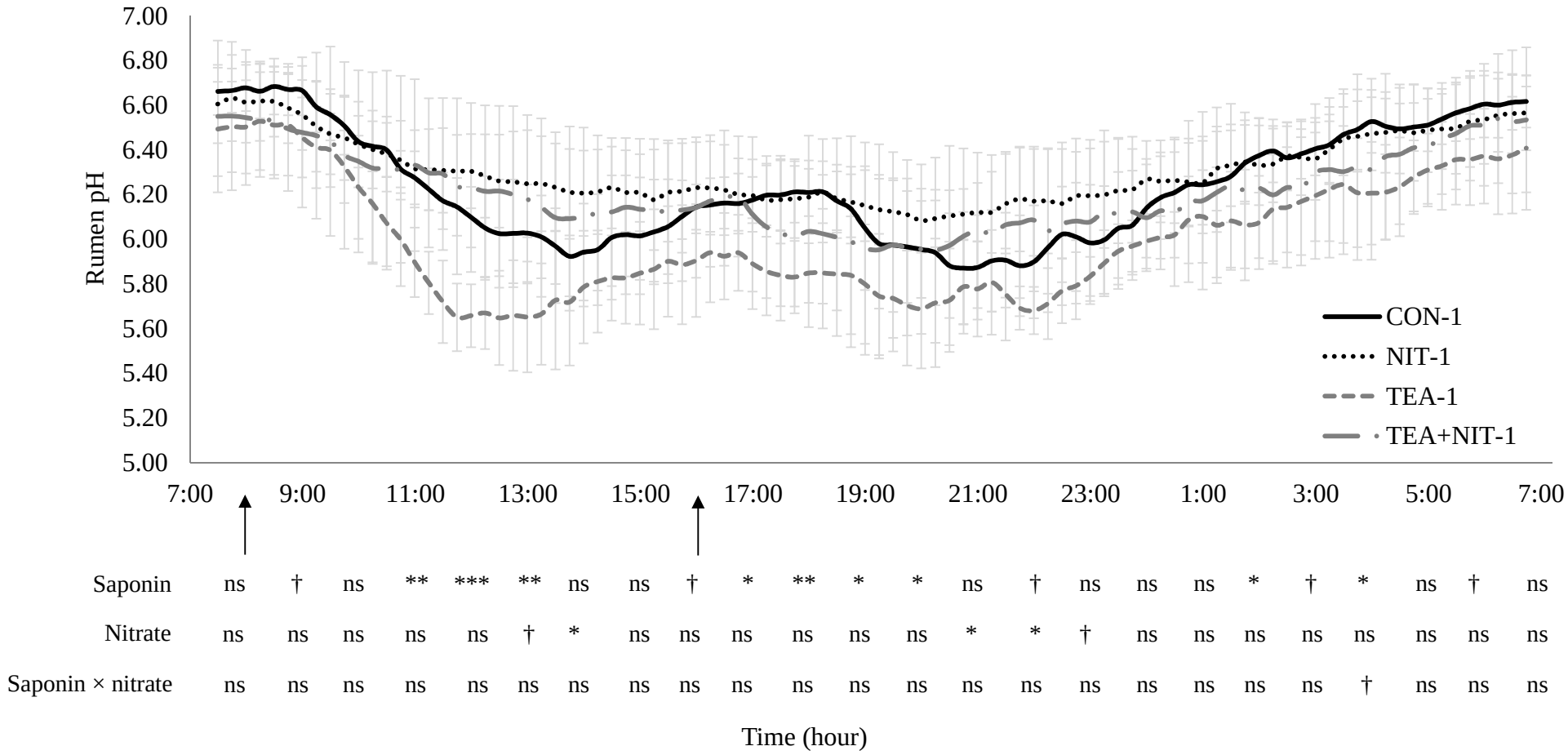
**Supplementary File – for Online Publication Only**



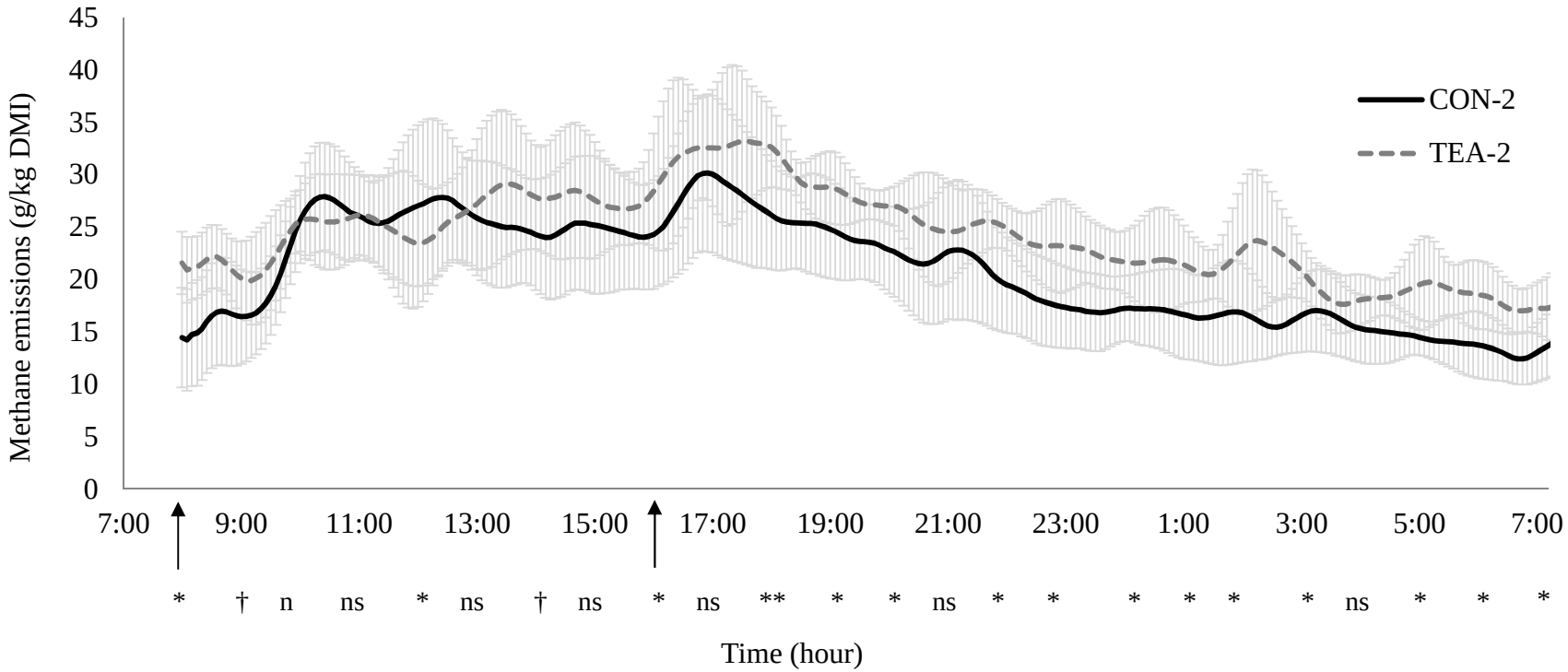

**Supplementary Figure S1.** Boxplot of blood metHb levels of non-lactating cows fed diets containing 2.3% nitrate with or without 0.5% tea saponin (n = 8; trial 1). The box represents the quartiles with the median at the center and the vertical lines represent the maximum and minimum value within 1.5 interquartile range of the higher and lower quartile, respectively. Values greater than 1.5 interquartile range are considered as outliers and are identified with a star. Blood was analyzed during the three weeks adaptation period, the arrow indicates the start of the measurement period.




**Supplementary Figure S2.** Daily $CH_4$ production pattern of non-lactating cows fed diets containing 2.3% nitrate with or without 0.5% tea saponin (n = 4; trial 1). Errors bars indicate s.d. Treatments consisted in diet control (CON-1), diet CON-1 containing 2.3% nitrate (NIT-1), diet CON-1 containing 0.5% tea saponin (TEA-1) and diet CON-1 containing 0.5% tea saponin and 2.3% nitrate (TEA+NIT-1). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison († = $P$<0.10; * = $P$<0.05; ** = $P$<0.01; *** = $P$<0.001) between treatments: saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); saponin × nitrate = interaction between main effects of tea saponin and nitrate.




**Supplementary Figure S3.** Daily pattern of rumen pH of non-lactating cows fed diets containing 2.3% nitrate with or without 0.5% tea saponin (n = 4; trial 1). Errors bars indicate s.d. Treatments consisted in diet control (CON-1), diet CON-1 containing 2.3% nitrate (NIT-1), diet CON-1 containing 0.5% tea saponin (TEA-1) and diet CON-1 containing 0.5% tea saponin and 2.3% nitrate (TEA+NIT-1). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison († = $P<0.10$; * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$) between treatments: saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); saponin × nitrate = interaction between main effects of tea saponin and nitrate.




**Supplementary Figure S4.** Daily $CH_4$ production pattern of lactating cows fed a diet containing 0.5% tea saponin (n = 7; trial 2). Errors bars indicate s.d. Treatments consisted in diet control (CON-2) and diet CON-2 containing 0.5% tea saponin (TEA-2). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison between CON-2 and TEA-2 († = $P$<0.10; * = $P$<0.05; ** = $P$<0.01; *** = $P$<0.001).

# STEP 4: Dietary nitrate inhibits rumen methanogenic archaea without influencing genes coding for microbial nitrate or nitrite reductases

## Objective

To study the effect of $CH_4$-mitigating strategies acting on $H_2$ production (lipids from linseed or saponin from tea, toxic effect towards protozoa) and $H_2$ utilization (nitrate from calcium nitrate, $H_2$-sink through nitrate reduction to nitrite and ammonia) on abundance, activity and diversity of rumen microbiota from non-lactating cows.

## Experimental approach

4 non-lactating cows → 2 × 2 Factorial design → FD **1**

**CON**: 50% hay + 50% pelleted concentrate
**NIT**: CON + 2.3% nitrate (from calcium nitrate)
**LIN**: CON + 2.6% added lipids (from linseed oil)
**LIN+NIT**: CON + 1.0% added lipids + 2.3% nitrate

4 non-lactating cows → 2 × 2 Factorial design → FD **2**

**CON**: 50% hay + 50% pelleted concentrate
**NIT**: CON + 2.3% nitrate (from calcium nitrate)
**TEA**: CON + 0.5% saponin (from tea)
**TEA+NIT**: CON + 0.5% saponin + 2.3% nitrate

Rumen samples taken in wk 5 (FD1) or wk 4 (FD2) 3 h after morning feeding

Total nucleic acids extraction (DNA and RNA) and cDNA synthesis

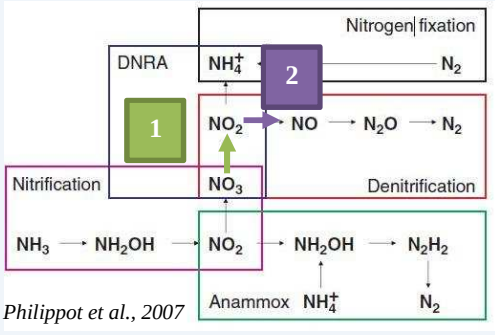
Abundance (DNA) and gene expression (cDNA) analysis by real-time PCR:

- Total bacteria (*rrs*) & methanogens (*mcrA*)
- Nitrate reducing bacteria (*napA* and *narG* /1)
- Nitrite reducing bacteria (*nirK* /2)

Diversity (DNA and cDNA) analysis with MiSeq, Illumina:

- Bacteria, protozoa, methanogens, fungi

**In progress (Annex 1)**




*Philippot et al., 2007*

## Main results


- Total rumen bacteria abundance was similar between diets.
- Methanogens abundance was reduced by nitrate- and lipids- containing diets in FD1 but not in FD2. Methanogens activity was reduced by 2.3 folds on average in diets including nitrate (NIT and LIN+NIT in FD1; NIT and TEA+NIT in FD2).
- Relative abundance of *napA*, *narG* and *nirK* DNA copies were similar between diets in FD1 and FD2. Only *narG* activity was detected without difference between dietary treatments.

## Conclusion

Lipids from linseed, saponin from tea, nitrate and their association (linseed plus nitrate and linseed plus tea saponin) act differently on rumen microbiota. Linseed reduced methanogens abundance, which may be explained by a toxic effect of fatty acids. Tea saponin did not affect targeted microbial population. Nitrate fed alone or in association with linseed or tea saponin did not affect nitrate and nitrite reducing bacteria, but had a toxic effect towards abundance and activity of methanogens, probably linked to nitrite toxicity. Further work is in progress to assess the effect of these three dietary treatments on diversity of rumen microbiota.

# Dietary nitrate inhibits rumen methanogenic archaea without influencing genes coding for microbial nitrate or nitrite reductases

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*In progress*

**Abstract**

This work assessed the effect of nitrate fed alone or in association with linseed or tea saponin on the abundance and activity of rumen bacteria, methanogens and nitrate and nitrite reductases. Two 2 × 2 factorial design experiments (FD1 and FD2) were performed using four non-lactating cows each. Diets were: 1) control, 2) control with 2.3% nitrate, 3) control with 4% linseed oil (FD1) or 0.77% tea saponin (FD2), and 4) control with 2.3% nitrate and 4% linseed oil (FD1) or 0.77% tea saponin (FD2). Rumen content was sampled after morning feeding at the end of each experimental period. Extracted nucleic acids were used for microbial quantification and gene expression analysis by qPCR. Targeted genes were: *rrs* (total bacteria), *mcrA* (methanogens), *narG*, *napA* and *nirK* (nitrate and nitrite reductase). Total bacteria abundance was similar among diets. Nitrate fed alone or in association with linseed reduced methanogens abundance and *mcrA* expression (FD1). Nitrate fed alone or in association with tea saponin only reduced *mcrA* expression (FD2). Abundance and expression of *narG*, *napA* and *nirK* were unaffected by diets. Dietary nitrate inhibited rumen methanogens but did not affect microbial genes coding for nitrate or nitrite reductases.

**Keywords:** Methanogens; Nitrate; Nitrate reductase; Nitrite reductase; Rumen

**Background**

We found a methane ($CH_4$)-mitigating effect of nitrate (2.3% in dry matter, DM) fed alone or in association with linseed oil (2.6% added fat in DM, [8]) or tea saponin (0.5% saponin in DM; Guyader et al., personal communication) in non-lactating cows. The predominant pathway of nitrate metabolism in the rumen is the reduction of nitrate to nitrite and nitrite to ammonia which consumes four moles of hydrogen ($H_2$) [14] thus reducing $H_2$ availability for methanogens. Another pathway of nitrate reduction consists in denitrification to produce gaseous nitrous oxide ($N_2O$) [23]. These mechanisms require the presence of bacteria known to reduce nitrate or nitrite such as *Selenomonas ruminantium*, *Veillonella parvula* and *Wolinella succinogenes* [11]. However, the quantity of these rumen bacteria was not affected when nitrate was supplemented to goats (1% in DM; [2]) or steers (1.2% in DM; [15]). Nevertheless, the effect of nitrate supplementation on both abundance and expression of universal genes coding for nitrate reductases in the rumen has never been assessed. In addition, whereas $N_2O$ has been detected in eructated gaseous emissions of dairy cattle supplemented with 2.1% nitrate in DM [22], the abundance and expression of genes targeting nitrite reductases in the rumen have never been studied.

Sheep fed a corn silage-based diet had reduced abundance of rumen methanogens when supplemented with 2.1% nitrate (in DM) [26]. We also observed that nitrate supplementation induced a rise of dissolved $H_2$ concentrations in the rumen of cows following ingestion [9]. These results suggest that nitrate may not only act as a $H_2$-sink but may also have a direct inhibiting effect on rumen methanogens. Nevertheless, the abundance and activity of methanogens in the rumen of cattle supplemented with nitrate is unknown.

The objective of this work was to assess the effect of nitrate fed alone or in association with linseed or tea saponin on i) the abundance and activity of methanogens, and ii) the abundance and expression of microbial genes targeting nitrate and nitrite reductases in the rumen of cows.

**Materials and methods**

The experiment was conducted at the animal experimental facilities of INRA's Herbivores Research Unit (UERT, Saint-Genès-Champanelle, France) from January to June 2013. All procedures involving animals were conducted in accordance with the French Ministry of Agriculture guidelines for animal research, and all applicable European guidelines and regulations on animal experimentation. The experiment was approved by the Auvergne regional ethic committee for animal experimentation, approval number CE50-12.

*Animals, experimental design and feeding management*

Eight non-lactating Holstein cows were separated into two groups conducted in parallel according to 2 × 2 factorial designs. Within each experiment, four cows were randomly assigned to four dietary treatments during 5-week experimental periods. In factorial design 1 (FD1), diets were on a DM basis: 1) control diet (CON, 50% natural grassland hay and 50% concentrate), 2) control diet with 4% linseed oil (LIN; 2.6% added fat), 3) control diet with 3% calcium nitrate (NIT; 2.3% nitrate), and 4) control diet with 4% linseed oil plus 3% calcium nitrate (LIN+NIT; 2.6% added fat plus 2.3% nitrate) [8]. In factorial design 2 (FD2), diets were on a DM basis: 1) control diet (CON, 50% natural grassland hay and 50% concentrate), 2) control diet with 0.77% tea saponin (TEA; 0.5% saponin), 3) control diet with 3% calcium nitrate (NIT; 2.3% nitrate), and 4) control diet with 0.77% tea saponin plus 3% calcium nitrate (TEA+NIT; 0.5% saponin plus 2.3% nitrate). Chemical composition of diets CON and NIT were similar between the two experiments.

*Rumen content sampling for microbial analysis*

At the end of each experimental period, rumen contents of cows were sampled over two days. Whole rumen content samples (200 g) were taken, through the cannula, from multiple sites within the rumen. Sampling was done 3 h after the morning feeding when $CH_4$ emissions differences between diets measured on the same animals were maximal [8]. A part of each sample (~30 g) was mixed with 30 mL ice cold PBS pH 6.8 and homogenized using a Polytron grinding mill (Kinematica GmbH, Steinhofhalde, Switzerland) for three cycles of 1 min with intervals of 1 min on ice. Then, approximately 0.5 g were transferred to a 2.5 mL Eppendorf tube and mixed with 1 mL of RNA*later*® Stabilization Solution (Applied Biosystems, Austin, TX, USA). Tubes were immediately stored at -80°C until total nucleic extractions which were done within 3 months of storage. Remaining rumen samples were used to determine DM of rumen content (103°C for 24h).

*Total nucleic acids extraction and cDNA synthesis*

Total nucleic acids (DNA and RNA) were co-extracted from all samples by bead-beating and phenol-chloroform extraction followed by saline-alcohol precipitation [24]. The yield and purity of extracted DNA and RNA were assessed using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), by measuring the absorbance intensity at 260 nm and the absorbance ratio 260/280, respectively. RNA integrity was estimated with an Agilent RNA 6000 Nano Kit on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa

Clara, CA, USA) according to the manufacturer's instructions. RNA Integrity Number (RIN) and the ratio between ribosomal RNA (rRNA) 23S/16S were calculated using the Software 2100 Expert, version B 02.08. SI648 (SR2; Agilent Technologies, Waldbronn, Germany).
Following extraction and quality assessment, RNA was reverse transcribed using the Reverse Transcriptase Kit with random primers (Promega, Madison, USA) according to manufacturer's instructions, on a T-100 thermocycler (BioRad, Hercules, USA). Both DNA and cDNA were stored at -20°C before subsequent analyses within 2 months following DNA extraction and cDNA synthesis.

*Quantification and gene expression of microbial communities*

Samples from each cow from the two sampling days of each experimental period were pooled by mixing an equal quantity of DNA or cDNA reaction volume, respectively. Quantification of gene targets were performed on microbial DNA and cDNA by quantitative PCR (qPCR) using a Step One Plus apparatus (Applied Biosystems, Villebon sur Yvette, France). Reactions were run in triplicate in 96-well plates, using 15.5 µL of 1X Takara SYBR Premix Ex Taq (Lonza, France), 0.25 µmoles of each forward and reverse primer and 20 ng of DNA or 2 µL of cDNA in a final volume of 20 µL. In this study, we used universal primers targeting the bacterial *rrs* gene and methanogenic specific primers, which were both designed for the rumen ecosystem. We also used universal primers to target nitrate and nitrite reductase genes; however, these pairs of primers were designed based on sequences recovered from non-rumen ecosystems. Primers description, average amplification efficiency, slope and $R^2$ of qPCR are described in Table 1, as required by MIQE guidelines for PCR [4]. Negative controls without templates were run in each assay to assess overall specificity.
Abundance of total bacteria (based on *rrs* DNA copies) was assessed using absolute quantification. Standard curve [19], amplification and melting curve were carried out as previously described [7]. Abundance of methanogenic archaea (based on *mcrA* DNA copies) was also assessed using absolute quantification, with standard curve prepared as previously described [19]. Level of expression of the functional *mcrA* gene (based on *mcrA* cDNA copies) was assessed using relative quantification with *rrs* cDNA copies used as reference. For both *mcrA* gene quantification and expression analyses, amplification and melting curve programs were performed as previously described [5].
Copy number and level of expression of genes involved in nitrate and nitrite reduction were analyzed by targeting two genes coding for a membrane-bound (*narG*) and a periplasmic (*napA*) nitrate reductase commonly found in bacteria from anaerobic estuarine sediments [25]

and one gene coding for a nitrite reductase found in bacteria from soil (*nirK* [10]). The presence of these three genes in rumen metagenomes was checked using the metagenomics RAST server [18]: *narG* and *napA* were already described in the rumen, whereas *nirK* was not reported. The qPCR program was the same as for total bacteria. Abundance (based on DNA copies) and activity (based on cDNA copies) of these genes were assessed using relative quantification with *rrs* as the reference gene (DNA *rrs* or cDNA *rrs*).

**Table 1** Description of primers (sequences, product size, average amplification efficiency, slope and $R^2$) used for quantifying abundance and activity of total bacteria, methanogenic archaea and nitrate and nitrite reductases by qPCR

| Organism or enzyme | Target gene | Primer set | Primer sequences 5'-3' | Product size (bp) | Efficiency | Slope | $R^2$ |
|---|---|---|---|---|---|---|---|
| Total bacteria [7] | *rrs* | 520 F<br>799 R2 | AGCAGCCGCGGTAAT<br>CAGGGTATCTAATCCTGTT | 280 | 1.88 | -3.64 | 0.999 |
| Methanogenic archaea [5] | *mcrA* | q*mcrA* F<br>q*mcrA* R | TTCGGTGGATCDCARAGRGC<br>GBARGTCGWAWCCGTAGAATCC | 140 | 1.96 | -3.43 | 0.995 |
| Nitrate reductase [25] | *napA* | *napA* 1F<br>*napA* 1R | GTYATGGARGAAAAATTCAA<br>GARCCGAACATGCCRAC | 111 | 2.01 | -3.29 | 0.999 |
| | *narG* | *narG* 2F<br>*narG* 2R | CTCGAYCTGGTGGTYGA<br>TTYTCGTACCAGGTSGC | 89 | 1.97 | -3.39 | 1.000 |
| Nitrite reductase [10] | *nirK* | *nirK*876 F<br>*nirK*1040 R | ATYGGCGGVAYGGCGA<br>GCCTCGATCAGRTTRTGGTT | 165 | 1.99 | -3.34 | 0.999 |

*Quantitative PCR calculations and statistical analysis*

Technical triplicates were averaged while checking overlaying of amplification plots at threshold cycle ($C_t$) value. Absolute quantification of total bacteria and methanogenic archaea were expressed as $\log_{10}$ *rrs* or *mcrA* copies/g DM rumen content, respectively. Relative quantification and expression of genes coding for nitrate (*narG* and *napA*) or nitrite (*nirK*) reductases, as well as gene expression of *mcrA* were assessed by the $C_t$ of the qPCR and the $2^{-\Delta Ct}$ method [16]:

$$2^{-\Delta Ct} = 2^{-(Ct\ target\ gene - Ct\ rrs)}$$

Data were analyzed using the MIXED procedure of SAS (Version 9.2; SAS Institute, 2009) and for the two experiments separately. The statistical model included the random effect of cow (n = 4) and fixed effects of period (n = 4), nitrate (CON and LIN *versus* NIT and

LIN+NIT in FD1; CON and TEA *versus* NIT and TEA+NIT in FD2), linseed (CON and NIT *versus* LIN and LIN+NIT in FD1), tea saponin (CON and NIT *versus* TEA and TEA+NIT in FD2) and the interaction linseed × nitrate (FD1) or tea saponin × nitrate (FD2). Data were considered significant at $P \leq 0.05$. Trends were discussed at $0.05 < P \leq 0.1$. Least square means are reported throughout.

**Results**

Total nucleic acids were extracted with similar yields and purity for the 2 experiments. Electropherograms obtained for RNA integrity analysis presented two peaks corresponding to the 16S and 23S rRNA. Diets did not affect RIN which averaged 7.30 and 7.24 for FD1 and FD2, respectively.

*Abundance and activity of total bacteria and methanogens*

Diets did not change abundance of total bacteria that averaged 7.31 and 7.45 $\log_{10}$ *rrs* copies/g DM rumen content for FD1 and FD2, respectively (Tables 2 and 3). For control diets, abundance of methanogens was similar between the two experiments. In FD1, abundance of methanogens was reduced by nitrate-containing diets (NIT and LIN+NIT; 5.01 $\log_{10}$ *mcrA* copies/g DM rumen content on average) as compared to CON and LIN (5.18 $\log_{10}$ *mcrA* copies/g DM rumen content on average; $P=0.01$). Linseed-containing diets (LIN and LIN+NIT) also tended to reduce abundance of methanogens ($P<0.10$). Inversely, methanogens abundance was similar among diets in FD2.

Expression of *mcrA* was reduced by nitrate-containing diets for both experiments ($P<0.05$; Tables 2 and 3). The level of *mcrA* expression with NIT and LIN+NIT compared to CON and LIN was reduced by 2.5 folds in FD1. Similarly, the level of *mcrA* expression was reduced by 2.1 folds with NIT and TEA+NIT compared to CON and TEA in FD2.

**Table 2** Abundance of total bacteria, and abundance and activity of methanogenic archaea in the rumen of non-lactating cows supplemented with nitrate fed alone or in association with linseed oil (FD1, n = 4)

| | Diet[1] | | | | | *P*-Value[2] | | |
|---|---|---|---|---|---|---|---|---|
| Item | CON | NIT | LIN | LIN+NIT | SEM | Nitrate | Linseed | Linseed × nitrate |
| Total bacteria (*rrs*) | | | | | | | | |
| Concentration ($log_{10}$ copies/g DM rumen content) | 7.44 | 7.24 | 7.27 | 7.27 | 0.056 | 0.13 | 0.23 | 0.14 |
| Methanogenic archaea (*mcrA*) | | | | | | | | |
| Concentration ($log_{10}$ copies/g DM rumen content) | 5.30 | 4.97 | 5.05 | 5.05 | 0.056 | 0.01 | 0.06 | 0.01 |
| Activity ($2^{-\Delta Ct} \times 10^6$) | 23.91 | 10.49 | 21.54 | 8.19 | 3.384 | 0.01 | 0.51 | 0.99 |

[1]CON = control; NIT = diet CON including 2.3% nitrate from calcium nitrate; LIN = diet CON including 2.6% added fat from linseed oil; LIN+NIT = diet CON including 2.6% added fat from linseed oil plus 2.3% nitrate from calcium nitrate.

[2] Linseed = main effect of linseed oil (CON and NIT *versus* LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN *versus* NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed oil and nitrate.

**Table 3** Abundance of total bacteria, and abundance and activity of methanogenic archaea in the rumen of non-lactating cows supplemented with nitrate fed alone or in association with tea saponin (FD2, n = 4)

| | Diet[1] | | | | | *P*-Value[2] | | |
|---|---|---|---|---|---|---|---|---|
| Item | CON | NIT | TEA | TEA+NIT | SEM | Nitrate | Saponin | Saponin × nitrate |
| Total bacteria (*rrs*) | | | | | | | | |
| Concentration ($log_{10}$ copies/g DM rumen content) | 7.44 | 7.43 | 7.37 | 7.54 | 0.066 | 0.24 | 0.78 | 0.19 |
| Methanogenic archaea (*mcrA*) | | | | | | | | |
| Concentration ($log_{10}$ copies/g DM rumen content) | 5.37 | 5.38 | 5.24 | 5.47 | 0.090 | 0.24 | 0.80 | 0.29 |
| Activity ($2^{-\Delta Ct} \times 10^6$) | 18.67 | 7.40 | 16.08 | 8.28 | 4.463 | 0.004 | 0.70 | 0.44 |

[1]CON = control; NIT = diet CON including 2.3% nitrate from calcium nitrate; TEA = diet CON including 0.5% saponin from tea; TEA+NIT = diet CON including 0.5% saponin from tea.

[2] Saponin = main effect of tea saponin (CON and NIT *versus* TEA and TEA+NIT); Nitrate = main effect of nitrate (CON and TEA *versus* NIT and TEA+NIT); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

*Quantification and expression of genes coding for nitrate or nitrite reductases*

Relative abundance of *napA*, *narG* and *nirK* DNA copies were similar between diets for both experiments (Tables 4 and 5). In FD1, the $2^{-\Delta Ct}$ values for DNA copies of *napA*, *narG* and *nirK* averaged 0.77, 10.06 and 13.40, respectively. These values averaged 1.61, 15.26 and 24.04, respectively in FD2. Expression of *napA* and *nirK* genes was below the detection limits. Expression of *narG* was detected at similar levels between all diets: the $2^{-\Delta Ct}$ values were equal to 1.85 and 1.31 in FD1 and FD2, respectively.

**Table 4** Abundance and activity of nitrate (*napA* and *narG*) and nitrite (*nirK*) reductases in the rumen of non-lactating cows supplemented with nitrate fed alone or in association with linseed oil (FD1, n = 4)

| | Diet[1] | | | | | *P*-Value[2] | | |
|---|---|---|---|---|---|---|---|---|
| Item[2] | CON | NIT | LIN | LIN+NIT | SEM | Nitrate | Linseed | Linseed × nitrate |
| Nitrate reductase (*napA*) | | | | | | | | |
| Concentration ($2^{-\Delta Ct} \times 10^6$) | 0.79 | 0.75 | 0.82 | 0.73 | 0.123 | 0.61 | 0.96 | 0.84 |
| Activity ($2^{-\Delta Ct} \times 10^6$) | <LD | <LD | <LD | <LD | -- | -- | -- | -- |
| Nitrate reductase (*narG*) | | | | | | | | |
| Concentration ($2^{-\Delta Ct} \times 10^6$) | 10.80 | 10.05 | 10.42 | 8.96 | 1.281 | 0.42 | 0.58 | 0.78 |
| Activity ($2^{-\Delta Ct} \times 10^6$) | 1.90 | 2.09 | 1.54 | 1.87 | 0.474 | 0.60 | 0.56 | 0.88 |
| Nitrite reductase (*nirK*) | | | | | | | | |
| Concentration ($2^{-\Delta Ct} \times 10^6$) | 14.39 | 16.20 | 12.19 | 10.83 | 2.173 | 0.92 | 0.13 | 0.49 |
| Activity ($2^{-\Delta Ct} \times 10^6$) | <LD | <LD | <LD | <LD | -- | -- | -- | -- |

<LD = below limit of detection

[1]CON = control; NIT = diet CON including 2.3% nitrate from calcium nitrate; LIN = diet CON including 2.6% added fat from linseed oil; LIN+NIT = diet CON including 2.6% added fat from linseed oil plus 2.3% nitrate from calcium nitrate.

[2] Linseed = main effect of linseed oil (CON and NIT *versus* LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN *versus* NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed oil and nitrate.

**Table 5** Abundance and activity of nitrate (*napA* and *narG*) and nitrite (*nirK*) reductases in the rumen of non-lactating cows supplemented with nitrate fed alone or in association with tea saponin (FD2, n = 4)

| | Diet[1] | | | | | *P*-Value[2] | | |
|---|---|---|---|---|---|---|---|---|
| Item[2] | CON | NIT | TEA | TEA+NIT | SEM | Nitrate | Saponin | Saponin × nitrate |
| Nitrate reductase (*napA*) | | | | | | | | |
| Concentration ($2^{-\Delta Ct} \times 10^6$) | 1.33 | 1.83 | 1.77 | 1.52 | 0.313 | 0.54 | 0.74 | 0.10 |
| Activity ($2^{-\Delta Ct} \times 10^6$) | <LD[2] | <LD | <LD | <LD | -- | -- | -- | -- |
| Nitrate reductase (*narG*) | | | | | | | | |
| Concentration ($2^{-\Delta Ct} \times 10^6$) | 14.22 | 16.18 | 16.44 | 14.18 | 1.503 | 0.92 | 0.94 | 0.21 |
| Activity ($2^{-\Delta Ct} \times 10^6$) | 1.31 | 1.45 | 1.07 | 1.41 | 0.276 | 0.31 | 0.55 | 0.66 |
| Nitrite reductase (*nirK*) | | | | | | | | |
| Concentration ($2^{-\Delta Ct} \times 10^6$) | 22.77 | 25.92 | 25.26 | 22.21 | 2.329 | 0.98 | 0.74 | 0.13 |
| Activity ($2^{-\Delta Ct} \times 10^6$) | <LD | <LD | <LD | <LD | -- | -- | -- | -- |

<LD = below limit of detection

[1]CON = control; NIT = diet CON including 2.3% nitrate from calcium nitrate; TEA = diet CON including 0.5% saponin from tea; TEA+NIT = diet CON including 0.5% saponin from tea.

[2] Saponin = main effect of tea saponin (CON and NIT *versus* TEA and TEA+NIT); Nitrate = main effect of nitrate (CON and TEA *versus* NIT and TEA+NIT); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

## Discussion

*Absence of dietary treatment effect on total bacteria concentration*

The abundance of total bacteria in the rumen of non-lactating cows fed nitrate (2.3% in DM) alone or in association with linseed (2.6% added fat in DM) or tea saponin (0.5% saponin in DM) was similar between diets. Our results are in accordance with the literature since nitrate (2.1% in DM) and lipids from soybean (up to 4.4% added fat in DM) fed individually to sheep [26] or steers [6] did not affect total abundance of ruminal bacteria. The effect of tea saponin on total bacteria has never been studied, but Mao et al., (2010) [17] reported no effect on the concentration of cellulolytic bacteria (*Ruminococcus flavefaciens* and *Fibrobacter succinogenes*) in the rumen of sheep supplemented with the same plant extract at a similar dose (0.5% tea saponin in DM). To our knowledge, this is the first report showing that there was no additional effect on rumen total bacteria abundance when combining nitrate with linseed or tea saponin.

*Nitrate toxicity on rumen methanogens*

In our study, 2.3% nitrate fed alone reduced $CH_4$ emissions of non-lactating cows by 25% on average [8], slightly reduced *mcrA* DNA copies in FD1 (-0.17 $\log_{10}$ *mcrA* copies/g DM rumen content) and *mcrA* expression in the two experiments (-2.3 folds). The negative effect of nitrate on methanogens' abundance estimated by qPCR has already been highlighted in sheep supplemented with 2.1% nitrate (-0.7 $\log_{10}$/mL of rumen contents; [26]). The inhibitory effect of nitrate and other derivative N-compounds (nitrite, nitric oxide and nitrous oxide) on *Methanosarcina barkeri*, *Methanobacterium bryantii* and *Methanobacterium formicicum* has also been reported in *in vitro* experiments with soil and salt marsh sediments [3, 13]. However, the negative effect of nitrate on *mcrA* expression in the gastrointestinal tract of animals has never been reported before.

Nitrate is known to reduce $CH_4$ emissions of ruminants by acting as a $H_2$-sink during its reduction to nitrite and ammonia [14]. As a consequence, nitrate would have an indirect effect on methanogens activity by decreasing $H_2$ availability. According to our results, nitrate would also have a direct toxic effect on methanogens as suggested by the rise of dissolved $H_2$ concentration in the rumen and of gaseous $H_2$ emissions eructated during the 3 h following nitrate supplementation to sheep [26] and cows [9]. Then, as long as nitrate consumes $H_2$, rumen $H_2$ availability is low and methanogens activity decreases. When nitrate has been reduced, the derivative N-compounds act as methanogen inhibitors, and rumen dissolved $H_2$ concentrations and gaseous $H_2$ emissions increase. Similar findings have been reported in a previous work studying the effect of nitrate on methane production and fermentation by slurries of human fecal bacteria [1].

To our knowledge, the effect of associating nitrate to linseed or tea saponin on methanogens population has never been studied. While reducing $CH_4$ emissions by 17% [8], linseed tended to reduce the abundance of methanogens in the rumen of non-lactating cows (-0.09 $\log_{10}$ *mcrA* copies/g DM rumen content) without affecting their activity. This result confirms a previous *in vivo* experiment in which the ruminal concentration of methanogens in dairy cows, fed a corn silage-based diet supplemented with linseed (up to 5% added fat in DM) was significantly reduced 3 h after feeding (-0.47 $\log_{10}$ *mcrA* copies/µg DNA; [20]). In our study, we suggest that methanogens reduction with linseed is associated to a decrease in $H_2$ availability, as protozoa which are important $H_2$-producers in the rumen were reduced by 52% in LIN compared to CON [8]. Tea saponin did not change the abundance or activity of rumen methanogens. Our results strengthen previous observations [17, 27] and correlate with the absence of $CH_4$-mitigating effect of this plant extract supplemented to the same animals

(Guyader et al., personal communication). Diets LIN+NIT and TEA+NIT reduced methanogens abundance and activity to a similar extent than when NIT was fed alone, suggesting that the effect was due to nitrate alone. However, LIN+NIT fed to these same animals additively reduced $CH_4$ emissions (-32%; [8]).

*Absence of nitrate effect on microbial genes coding for nitrate and nitrite reductases*

By a culture-based approach, it was already reported that some rumen bacteria (*S. ruminantium, V. parvula* and *W. succinogenes*) can reduce nitrate to nitrite and ammonia [11]. Moreover, qPCR data showed that rumen abundance of *S. ruminantium* and *V. parvula* was not affected in goats fed with 1% nitrate in DM [2]. Similarly, *V. parvula* (*rrs* gene copy number) remained stable in steers supplemented with 1.2% of nitrate [15]. Inversely, the number of *W. succinogenes* increased considerably in the rumen of goats supplemented with 1% nitrate in DM (from less than $1.0 \times 10^2$ to $1.2 \times 10^3$ cells/mL) [2]. Based on the above information, we can affirm that the effect of nitrate supplementation on microbes involved in nitrate metabolism in ruminants remains unclear and needs more investigation.

The present paper is the first one to target particular genes coding for nitrate reductases for assessing the potential activity of nitrate reduction that covers both identified and not-yet identified nitrate-reducing rumen microbes. We focused on the abundance and activity of genes coding for membrane-bound (*narG*) and periplasmic (*napA*) nitrate reductases. We first confirmed the presence of these genes in the rumen ecosystem by interrogating published rumen metagenomes; these genes are also present in the genomes of S. *ruminantium*, *W. succinogenes* and *V. parvula* [18]. However, we cannot exclude that the abundance and expression of targeted genes may be linked with bacterial sediment ingested with feed.

Both nitrate reductase genes *narG* and *napA* were detected but their abundance was not affected by nitrate supplementation. These results confirm a previous work in which *narG* relative abundance from *S. ruminantium* was similar between steers receiving or not 1.2% nitrate in DM [15]. Expression of *narG* was also not affected by diets. The level of expression of *napA* was low suggesting that this gene was not expressed, or that the level of expression was below the detection limits.

A recent work reported that $N_2O$ emissions occurred when dairy cattle were fed up to 2.1% nitrate in DM [22], suggesting that rumen nitrate degradation may partially follow the denitrification pathway (nitrate to nitrite to nitric oxide to nitrous oxide) [12, 23]. In our experiment, abundance and activity of nitrite reductase, performing the reduction of nitrite to nitric oxide, were evaluated by monitoring *nirK*, which is found in bacteria from soil but not clearly annotated in published rumen metagenomes. Although this gene was detected in

rumen microbial DNA, its abundance was not affected by nitrate supplementation and, additionally, its level of expression was below the detection limits. Further work should assess the effect of nitrate supplementation on both $N_2O$ emissions and on the abundance and expression of other genes (e.g. *nirS*, [21]) known to be involved in the reduction of nitrite to $N_2O$.

**Concluding remarks**

We showed an inhibitory effect of dietary nitrate on the activity of rumen methanogens in non-lactating cows. Abundance and expression of *narG* and *napA* genes coding for nitrate reductases and *nirK* gene coding for a nitrite reductase were not affected by nitrate supplementation. Further work is required to assess the effect of nitrate on other nitrate and nitrite reductases which have been recently found within the rumen metagenome. The use of high throughput sequencing methods is in progress to assess the effect of dietary nitrate on the rumen microbiota diversity.

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# STEP 5: Dose response effect of nitrate on hydrogen distribution between rumen fermentation end-products: an *in vitro* approach

## Objective

1/ To study the dose response effect of nitrate on *in vitro* production of rumen fermentation end-products such as gas ($CH_4$ and $H_2$), VFA and microbial biomass (estimated from insoluble protein).
2/ To understand the $CH_4$-mitigating mechanisms of nitrate by estimating metabolic $H_2$ distribution between rumen fermentation end-products.

## Experimental approach

*In vitro* system → 2 repeated incubations → Dose response: CON **(50% hay + 50% concentrate)** + 0, 1, 2, 4 or 6 m*M* nitrate (from ammonium nitrate) — Exp **1**

→ 2 repeated incubations → Dose response: CON **(100% glucose)** + 0, 1, 2, 4 or 6 m*M* nitrate (from ammonium nitrate) — Exp **2**

| HOUR | 1 | … | 3 | … | 8 | … | 12 | … | 24 | … | 32 | … | 48 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kinetics of gas production (total, $CH_4$ and $H_2$; exp 1 and 2) | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ |
| pH, VFA and $NH_4^+$ concentrations (exp 1) | | | | | | | | | | | | | ■ |
| pH (48 h), kinetics of VFA, $NH_4^+$ and insoluble proteins concentrations (exp 2) | ■ | | ■ | | ■ | | ■ | | ■ | | ■ | | ■ |

Estimation of metabolic $H_2$ distribution between rumen fermentation end-products (mmoles):

- $H_2$ production = 2 × acetate + 2 × butyrate
- $H_2$ consumption = 4 × $CH_4$ + 1 × propionate + 4 × $NO_3^-$ + 0.41 × microbial biomass (insoluble protein)
- $H_2$ balance = $H_2$ production – $H_2$ consumption – $H_2$ emissions

## Main results

Exp **1** Exp **2**

- In exp 1 and 2, total gas and $CH_4$ production linearly decreased as nitrate doses increased. Nitrate reduced $CH_4$ production during the first 10 h of incubation. Hydrogen emissions were detected only with high doses of nitrate, after 10 h incubation.
- Rumen fermentation parameters including microbial biomass synthesis (calculated from insoluble protein concentration in exp 2) were poorly affected by nitrate.
- Estimated $H_2$ balance indicated that 23% (6m*M* nitrate; exp 1) of $H_2$ was not used for production of studied rumen fermentation end-products.

## Conclusion

Nitrate is an efficient $CH_4$-mitigating strategy, but with doses higher than 4 m*M*, *in vitro* fermentations were negatively affected. Estimation of $H_2$ distribution between studied rumen fermentation end-products suggest that nitrate enhances another $H_2$ consuming pathway.

# Dose response effect of nitrate on hydrogen distribution between rumen fermentation end-products: an *in vitro* approach

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**Abstract**

The objective of this work was to study the *in vitro* dose response effect of nitrate on hydrogen distribution between rumen fermentation end-products. Five nitrate concentrations (0, 1, 2, 4 and 6 mM) were tested in two *in vitro* experiments. In experiment 1, a mixture of hay and concentrate (50:50) was used to calculate efficiencies of methane reduction and to study differences between fermentation profiles. In experiment 2, glucose was used as the sole protein-free substrate to quantify the effect of nitrate dosage on microbial synthesis. In both experiments, two 48 h-incubations were carried out using bovine rumen contents as inoculum. Total gas production and composition was automatically analyzed throughout the incubations. In experiment 1, volatile fatty acids (VFA) and ammonium concentrations were analyzed from samples taken after 48-h incubation. In experiment 2, VFA, ammonium and insoluble protein concentrations were analyzed from samples collected at various time points. In experiment 1, total gas production was decreased with the highest dose of nitrate (P=0.019). Methane emissions tended to linearly decrease as nitrate doses increased (P=0.079). Kinetics of methane emissions showed that hydrogen removal via nitrate reduction occurred mainly during the first 10 h-incubation. The apparent yield of methane reduction relative to control incubations exceeded 100% with nitrate doses higher than 4 mM. Gaseous hydrogen production was similar between treatments, despite numerically higher hydrogen emissions for nitrate concentrations above 4 mM. Concentrations and proportions of VFA were not affected by treatments. Proportions of unaccounted hydrogen in total hydrogen produced were similar and positive for all treatments, despite a numerical increase as nitrate doses increased. Experiment 2 showed that insoluble protein concentrations were not affected by nitrate. In this *in vitro* work, we confirmed that nitrate acts as an electron acceptor in the rumen. We also suggest that nitrate or its reduced forms have a direct inhibiting effect towards methanogens, as indicated by the release of gaseous hydrogen and the high efficiencies of methane reduction.

**Highlights**

- Increasing nitrate dose linearly reduces methane emissions *in vitro*.
- High doses of nitrate inhibit overall gas production.
- High doses of nitrate do not reduce methane by only acting as an electron acceptor.
- Nitrate does not enhance microbial synthesis.

**Keywords:** hydrogen, *in vitro*, methane, microbial biomass, nitrate, rumen

## Introduction

In the rumen, hydrogen ($H_2$) is produced by bacterial and protozoal hydrogenases after the reoxidation of coenzymes or pyruvate generated during the synthesis of volatile fatty acids (VFA): the production of one mole acetate or butyrate generates two moles $H_2$ (Hegarty and Gerdes, 1999). Since an increased $H_2$ concentration inhibits the normal function of microbial enzymes in the rumen, $H_2$ disposal is essential. Most of the $H_2$ is used to reduce carbon dioxide ($CO_2$) to methane ($CH_4$) consuming 4 moles $H_2$ per mole $CH_4$ produced. Then, methanogenesis uses between 48 and 80% of $H_2$ (Czerkawski, 1986; Mills et al., 2001). Between 19 and 33% of $H_2$ is used for VFA synthesis, as one mole $H_2$ is required per mole propionate or valerate produced. And finally, 0.6 to 12% of $H_2$ is used for microbial growth, as 0.41 moles $H_2$ are required per kg of microbes.

Considering the importance of $H_2$ in $CH_4$ production by ruminants, several $CH_4$ mitigation strategies aimed at reducing the availability of $H_2$ for microbial $H_2$-users such as methanogens. One of these strategies is to supply nitrate ($NO_3^-$) to the animals' diet. This additive would act as an electron acceptor reducing the amount of $H_2$ formed by 4 molar equivalents of $H_2$ through its reduction to nitrite ($NO_2^-$) and ammonium ($NH_4^+$). A recent meta-analysis reported that 1% $NO_3^-$ added to the diet of cattle reduced $CH_4$ emissions by 10% on average (Lee and Beauchemin, 2014). However, the dose response effect of $NO_3^-$ on both $CH_4$ emissions and rumen fermentation has not been reported or studied, due to the risks of blood methemoglobinemia for animals supplemented with high doses of this additive (Lewis, 1951).

Recent work also highlighted that $NO_3^-$ reduced the number and/or activity of methanogens (Van Zijderveld et al., 2010; Guyader et al., 2014c), changed fermentation profile towards acetate production (Veneman et al., 2014) and increased dissolved $H_2$ concentration in the rumen (Guyader et al., 2014b) and $H_2$ emissions (Van Zijderveld et al., 2011). These results

suggest that $NO_3^-$ may not only act as an electron acceptor in the rumen and that its $CH_4$-mitigating effect may involve other mechanisms. The study of $H_2$ fluxes towards fermentation end-products such as methanogenesis, VFA synthesis and microbial biomass, with different doses of $NO_3^-$ may allow a better understanding of the effect of this additive in the ruminal environment.

The objective of this work was to deepen the understanding of the $CH_4$-mitigating mechanisms of $NO_3^-$ by studying its dose response effect on i) $CH_4$ emissions and microbial fermentation profile, and ii) the distribution of $H_2$ between fermentation end-products. Due to the risk of blood metHb for animals fed high doses of $NO_3^-$ (Lee and Beauchemin, 2014), an *in vitro* approach was favored and two experiments were carried out. In the first experiment, a hay and concentrate based substrate was used in order to get close to ruminants diet conditions. The apparent yield of $CH_4$ reduction with different $NO_3^-$ concentrations and their effects on fermentation profiles were studied. In the second experiment, glucose was used as the sole protein-free substrate to quantify the effect of $NO_3^-$ on microbial synthesis.

## Material and methods

Two *in vitro* experiments, each one consisting in two repeated incubations, were carried out at AgResearch Grasslands (Palmerston North, New Zealand) with a fully automated incubation system (Muetzel et al., 2014) using ammonium nitrate ($NH_4NO_3$) as the source of $NO_3^-$.

### *Design of experiments*

In experiment 1, a general purpose substrate (GP) was composed of a mixture of hay (500 g/kg), barley (290 g/kg), soybean (100 g/kg), molasses (100 g/kg), dicalcium phosphate (5.5 g/kg), salt (3 g/kg) and minerals and vitamins (1.5 g/kg) on a dry matter (DM) basis. The substrate was ground in a Wiley mill to pass a 1-mm screen. Treatments were: 1/ control (10 mg GP/ml medium), 2/ control plus 1 mM $NO_3^-$, 3/ control plus 2 mM $NO_3^-$, 4/ control plus 4 mM $NO_3^-$ and 5/ control plus 6 mM $NO_3^-$. Duplicate bottles for each treatment served as technical replicates.

In experiment 2, the substrate was composed of D-glucose (GLU) only. Treatments were: 1/ control (6.67 mg GLU/ml medium), 2/ control plus 1 mM $NO_3^-$, 3/ control plus 2 mM $NO_3^-$, 4/ control plus 4 mM $NO_3^-$ and 5/ control plus 6 mM $NO_3^-$. Four bottles were prepared per treatment: two bottles served as technical replicates for gas analysis whereas the two other ones served as technical replicates for frequent sample collection.

*Batch culture incubations*

Rumen contents were obtained from two ruminally fistulated cows. Within the two experiments, one different donor cow was used per incubation. The donor animals were kept on pasture at Grasslands animal facility. Samples were taken manually at 0830 h from the dorsal part of the rumen and were immediately placed in pre-warmed thermos and transported to the laboratory. The rumen contents were then strained through one layer of cheesecloth and diluted (20% v/v) with a warm (39°C), reduced and $CO_2$-saturated buffer solution (Mould et al., 2005). The medium was continuously subjected to a $CO_2$ stream and maintained at 39°C in a water bath before starting incubations. Treatments were incubated in pre-warmed (39°C) bottles filled with 60 ml buffered rumen fluid and purged with a $CO_2$ stream. Immediately after filling with the medium, the bottles were sealed with a butyl rubber stopper and placed on a shaker in an incubator and connected via a 23-gauge needle to the pressure sensor and valve setup. Samples were incubated for 48 h at 39°C.

*Sampling and gas measurement*

Before starting the incubation, a sample (1.8 ml) of the medium was collected for subsequent analysis of $NH_4^+$, VFA (experiment 1 and 2) and insoluble protein (experiment 2 only).

In the two experiments, kinetics of gas production and composition were determined throughout the incubations using an automated *in vitro* gas production system with a gas chromatograph attached for automatic $CH_4$ and $H_2$ analysis (Muetzel et al., 2014). In experiment 1, gas kinetics were determined in all bottles for 48 h. After 48 h incubation, the bottles were removed from the incubator, opened and pH was immediately measured. Samples (1.8 ml) were taken for subsequent analysis of $NH_4^+$ and VFA. In experiment 2, gas kinetics were determined for 48 h in two bottles out of the four bottles per treatment. The two other bottles were used for sampling (1.8 ml) after 1, 3, 8, 12, 16, 24, 32, and 48 h incubation for $NH_4^+$, VFA and insoluble protein analysis. At 48 h, the remaining bottles from the gas measurement were also collected as described above and pH was measured.

All samples were centrifuged (21,000×g at 4°C for 10 min). For $NH_4^+$ and VFA analysis, 0.9 ml of the supernatant was transferred in a micro centrifuge tube containing 0.1 ml of internal standard solution (19 mM ethyl butyrate in 20% (v/v) phosphoric acid), mixed well, and kept at -20°C over night. When insoluble protein concentration was analyzed, the remaining supernatant was discarded and the pellet was washed once with a saline solution (0.85% NaCl, w/v) and stored at -20°C until processed.

Thawed fluid samples were clarified by centrifugation (21,000×g at 4°C for 10 min) and 0.8 ml of the supernatant was transferred into a 2 ml crimp cap gas chromatography vial for VFA analysis and 0.1 ml was collected for $NH_4^+$ analysis. Volatile fatty acids were analyzed by gas chromatography (Attwood et al., 1998) and $NH_4^+$ was analyzed by a colorimetric method (Chaney and Marbach, 1962). Thawed pellets were suspended in 750 µl SDS (1%, w/v), using an Eppendorf MixMate at 2,000 rpm for 10 min. The samples were then heated to 100°C for 10 min to solubilize the proteins and then centrifuged (21,000×g at room temperature for 10 min). The supernatant (300 µl) was then transferred in a micro centrifuge tube containing 1 ml acetone (100%, w/w) for protein precipitation. After incubation (-30°C for 2 h), precipitated proteins were centrifuged (21,000×g at 4°C for 10 min) and washed with 600 µl acetone (75%, w/w). The final pellet was suspended in 300 µl SDS (1%, w/w) and the concentration of insoluble protein was determined using the Pierce BCA assay (Thermo Scientific, Rockford, USA).

*Calculations and statistical analyses*

For each incubation, a logistic model (France et al., 2000) was fitted to the 48 h gas production (total, $CH_4$ and $H_2$) data using least squares regression. The resulting logistic parameters were used to calculate gas production at 32 h. Yield of $CH_4$ reduction for a treatment was calculated as the ratio between observed $CH_4$ reduction for this treatment (ml/g) relative to its expected $CH_4$ reduction (ml/g) based on stoichiometry. Expected $CH_4$ reduction was calculated assuming that one mole $NO_3^-$ reduces $CH_4$ production by one mole.

Concentrations of $NH_4^+$ were corrected for the amount of $NH_4^+$ added from ammonium nitrate. Insoluble protein production was calculated by subtracting the initial insoluble protein concentrations in the medium from the concentrations of each bottle.

The VFA production data (at 48 and 32 h in experiments 1 and 2, respectively) were used to calculate net $H_2$ production (mmol/bottle) assuming that i) the formation of VFA was solely derived from carbohydrates fermentation to hexoses and pentoses; ii) the production of one mole acetate or one mole butyrate generates two moles $H_2$. Methane (at 32 h in both experiments) and propionate (at 48 and 32 h in experiments 1 and 2, respectively) production, $NO_3^-$ reduction and microbial biomass synthesis (at 32 h in experiment 2 only) were considered as $H_2$ consuming pathways. The amount of $H_2$ directed towards these pathways (mmol/bottle) was calculated considering that the synthesis of one mole $CH_4$ and propionate requires four and one mole $H_2$ respectively and that $NO_3^-$ reduction to $NH_4^+$ requires four

moles $H_2$. The amount of $H_2$ required for microbial biomass synthesis (mmol/bottle) was calculated using the following equation:

$$H_2 \text{ towards microbial biomass} = (ISP \times a \times b)/c$$

With ISP = insoluble protein concentrations (mg/ml), a = the volume of medium in each bottle (60 ml), b = the microbial requirement of $H_2$ when they grow without preformed amino acids (0.41 moles $H_2$/kg microbes; Mills et al., 2001) and c = the percentage of proteins in bacteria (54.46 g proteins/100g dry bacterial cells; Reichl and Baldwin, 1975). Finally unaccounted $H_2$ was calculated as the difference between estimated $H_2$ production (mmol/bottle) and $H_2$ consumption and gaseous $H_2$ (mmol/bottle).

Data from duplicate bottles were averaged for statistical analyses. The dose effect of $NO_3^-$ on gas production at 32 h (total gas, ml/g; $CH_4$, ml/g and % of total gas; $H_2$, ml/g and % of total gas; yield of $CH_4$ reduction), on fermentation parameters at 48 h for experiment 1 and 32 h for experiment 2 (pH; $NH_4^+$, mM; VFA, mmol/g; insoluble protein, mg/ml; acetate, propionate and butyrate, %; acetate/propionate and (acetate+butyrate)/propionate) and on $H_2$ metabolism ($H_2$ produced, consumed, emitted and unaccounted, mmol/bottles) was analyzed using the MIXED procedure of SAS (Version 9.4; SAS Institute, 2009). The statistical model included the fixed effect of $NO_3^-$ dosage (n = 5), and run (n = 2) was considered as a random effect. Differences between diets were tested using the PDIFF option. The effect of increasing level of $NO_3^-$ was assessed through linear, quadratic and cubic orthogonal contrasts using the CONTRAST statement of SAS. As $NO_3^-$ doses were not equidistant, the IML procedure was used to calculate coefficients for unequally spaced contrasts. Cubic effect was not significant and consequently its effect was not presented in the tables of results. Data were considered significant at $P<0.05$, and trends were discussed at $0.05<P<0.1$.

**Table 1** *In vitro* dose response effect of nitrate on gas production and composition after 32 h incubation, and on fermentation parameters after 48 h incubation with GP substrate (50% hay and 50% concentrate; experiment 1)

| | Nitrate dose (mM) | | | | | | P-Value[1] | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | 0 | 1 | 2 | 4 | 6 | SED | Dose | L | Q |
| Gas production and composition | | | | | | | | | |
| Total gas production (ml/g) | 259.7[a] | 253.4[a] | 248.4[a] | 236.5[a] | 191.6[b] | 20.15 | 0.019 | 0.003 | 0.114 |
| $CH_4$ production (ml/g) | 42.9[a] | 42.1[a] | 38.6[a] | 30.3[ab] | 18.7[b] | 9.67 | 0.079 | 0.013 | 0.460 |
| $CH_4$ production (% of total gas) | 16.5 | 16.6 | 15.5 | 12.6 | 9.2 | 3.75 | 0.229 | 0.044 | 0.589 |
| $H_2$ production (ml/g) | 0.26 | 0.25 | 0.26 | 1.17 | 1.90 | 1.160 | 0.460 | 0.114 | 0.628 |
| $H_2$ production (% of total gas) | 0.10 | 0.10 | 0.11 | 0.53 | 1.12 | 0.671 | 0.451 | 0.114 | 0.524 |
| Efficiency of $CH_4$ reduction (%) | -- | 26.8 | 80.0 | 119.1 | 152.5 | -- | -- | -- | -- |
| Fermentation parameters | | | | | | | | | |
| pH | 6.18[a] | 6.21[a] | 6.24[ab] | 6.27[b] | 6.29[b] | 0.066 | 0.042 | 0.007 | 0.295 |
| $NH_4^+$ (mM) | 30.31 | 27.95 | 30.37 | 29.75 | 32.42 | 2.984 | 0.666 | 0.354 | 0.501 |
| Total VFA (mmol/g) | 6.71 | 6.65 | 6.45 | 6.99 | 6.48 | 0.642 | 0.408 | 0.898 | 0.568 |
| Acetate (% of total VFA) | 60.2 | 61.1 | 61.7 | 62.1 | 62.2 | 3.66 | 0.838 | 0.353 | 0.645 |
| Propionate (% of total VFA) | 19.7 | 19.3 | 19.2 | 20.0 | 21.1 | 3.41 | 0.857 | 0.406 | 0.577 |
| Butyrate (% of total VFA) | 14.9 | 14.5 | 14.1 | 13.6 | 12.4 | 1.39 | 0.182 | 0.032 | 0.768 |
| Acetate/butyrate | 3.07 | 3.18 | 3.23 | 3.20 | 3.10 | 0.702 | 0.989 | 0.989 | 0.648 |
| (Acetate+butyrate)/propionate | 3.83 | 3.93 | 3.97 | 3.88 | 3.71 | 0.747 | 0.964 | 0.687 | 0.606 |

[a, b] Means in the same row with different superscripts differ ($P<0.05$).

[1] Orthogonal contrasts for L = linear and Q = quadratic effects.

**Results**

*Experiment 1*

After 32 h incubation with GP substrate, total gas production was not different between control and 1, 2 and 4 mM $NO_3^-$, and was reduced by 26% with 6 mM $NO_3^-$ (P=0.019; Table 1). Methane production expressed in ml/g tended to linearly decrease as $NO_3^-$ concentrations increased (P=0.079), whereas no difference between treatments was observed when $CH_4$ was expressed as a percentage of total gas produced. Yield of $CH_4$ reduction was lower than 100% with 1 and 2 mM $NO_3^-$, but exceeded 100% with concentrations higher than 4 mM. The kinetics of $CH_4$ production (ml/g) indicated that the decrease in $CH_4$ emissions occurred during the first 10 h of incubation (Figure 1) and after 10 h, the rate of $CH_4$ production appeared similar to the control treatment.

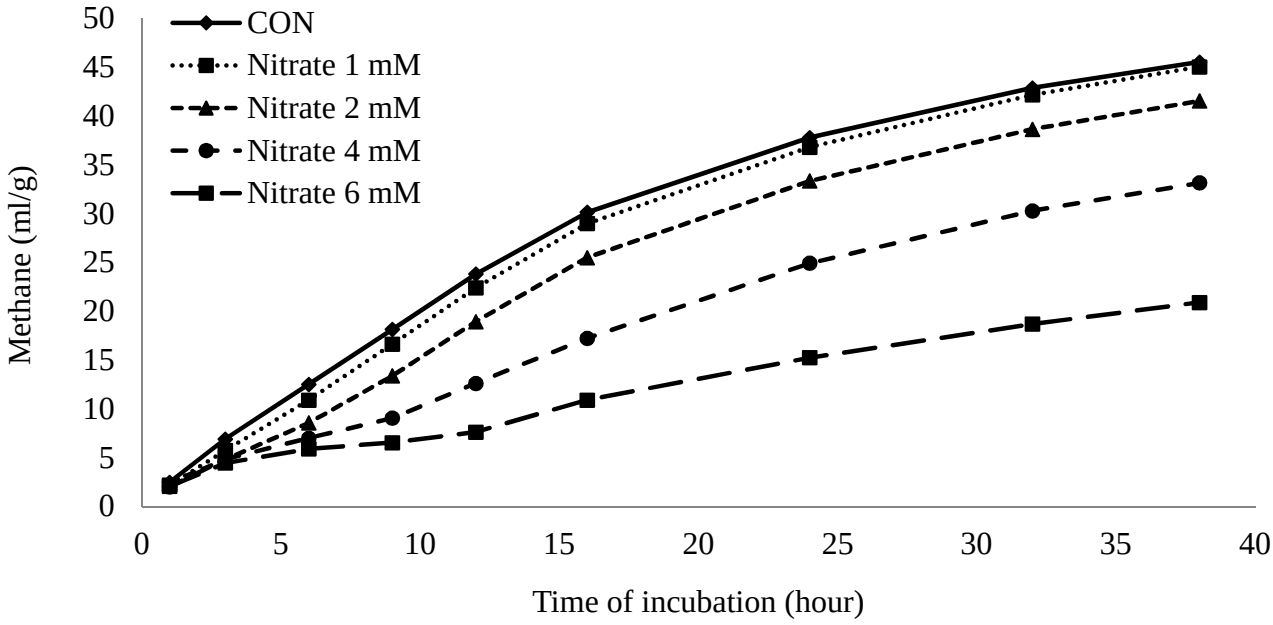


**Figure 1** *In vitro* dose response effect of nitrate on kinetics of methane production during 48 h incubation with GP substrate (50% hay and 50% concentrate; experiment 1)

Nitrate did not alter gaseous $H_2$ emissions expressed in ml/g or as a percentage of total gas produced (Table 1). However, more $H_2$ emissions occurred after 10 h and 15 h incubation for $NO_3^-$ doses of 4 and 6 mM, respectively (Figure 2).

The final pH linearly increased from 6.18 in the control to 6.29 for 6 mM $NO_3^-$ (P=0.042; Table 1). The concentrations of $NH_4^+$ and of total VFA production were not affected by $NO_3^-$ and averaged 30.2 mM and 6.7 mmol/g, respectively. Nitrate levels did not affect proportions of acetate, propionate, and butyrate, which averaged 61.5%, 19.9%, and 13.9%, respectively.

Consequently, the ratios acetate/propionate and (acetate+butyrate)/propionate were similar between treatments.

Total production and consumption of $H_2$ was not affected by treatments and averaged 5.11 and 4.43 mmol/bottle, respectively (Table 2). Unaccounted $H_2$ was positive and tended to increase with increasing levels of nitrate (from 11% in control to 23% with 6 mM $NO_3^-$; P=0.099).

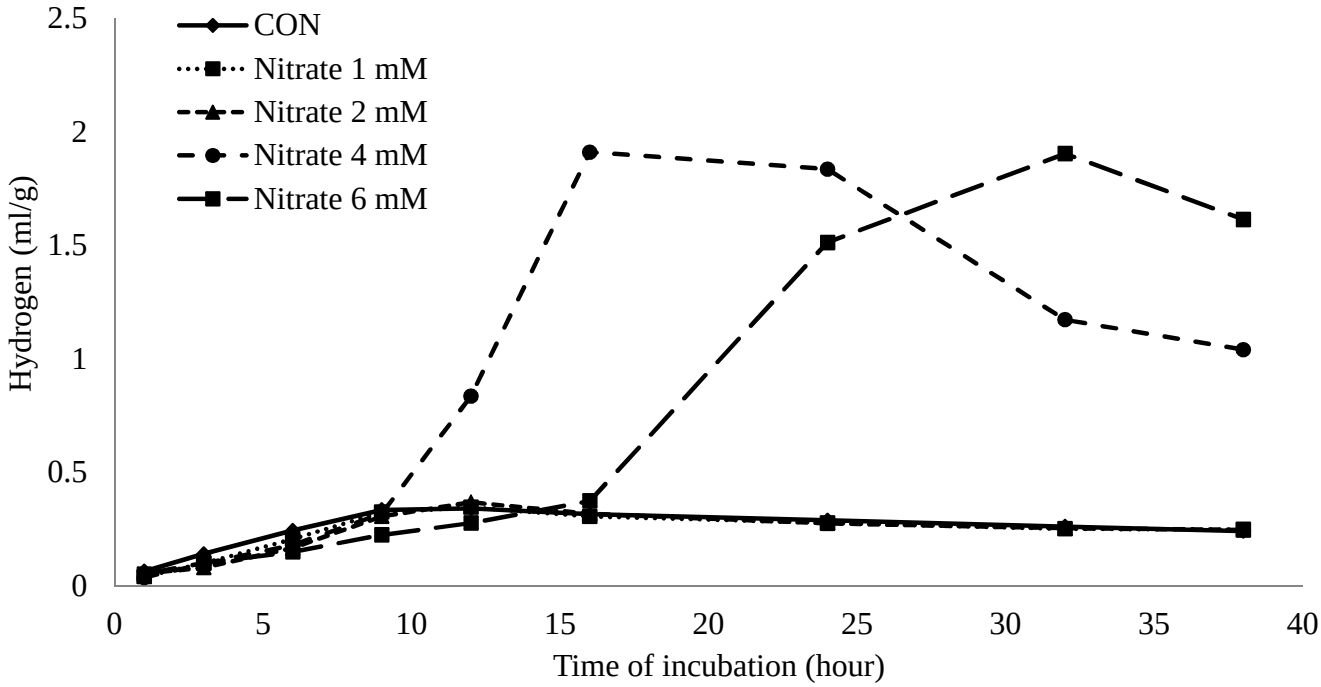


**Figure 2** *In vitro* dose response effect of nitrate on kinetics of hydrogen production during 48 h incubation with GP substrate (50% hay and 50% concentrate; experiment 1)

**Table 2** *In vitro* dose response effect of nitrate on calculated hydrogen production and distribution between fermentation end-products with GP substrate (50% hay and 50% concentrate; experiment 1)

| | Nitrate dose (mM) | | | | | | P-Value[1] | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | 0 | 1 | 2 | 4 | 6 | SED | Dose | L | Q |
| $H_2$ production (mmol/bottle) | | | | | | | | | |
| From acetate | 4.11 | 4.14 | 4.05 | 4.44 | 4.13 | 0.652 | 0.748 | 0.655 | 0.603 |
| From butyrate | 1.02[a] | 0.98[a] | 0.92[a] | 0.96[a] | 0.81[b] | 0.035 | 0.025 | 0.006 | 0.397 |
| Total | 5.13 | 5.12 | 4.97 | 5.39 | 4.94 | 0.649 | 0.612 | 0.887 | 0.524 |
| $H_2$ consumption (mmol/bottle) | | | | | | | | | |
| For methane | 3.89[a] | 3.83[a] | 3.51[a] | 2.75[ab] | 1.70[b] | 0.879 | 0.079 | 0.013 | 0.460 |
| For propionate | 0.67 | 0.65 | 0.63 | 0.70 | 0.68 | 0.052 | 0.457 | 0.342 | 0.644 |
| For nitrate reduction | 0.00 | 0.24 | 0.48 | 0.96 | 1.44 | 0.000 | -- | -- | -- |
| Total | 4.56 | 4.72 | 4.62 | 4.41 | 3.82 | 0.828 | 0.578 | 0.188 | 0.451 |
| $H_2$ emission (mmol/bottle) | 0.01 | 0.01 | 0.01 | 0.03 | 0.04 | 0.026 | 0.459 | 0.114 | 0.629 |
| $H_2$ unaccounted (mmol/bottle) | 0.56 | 0.39 | 0.35 | 0.96 | 1.08 | 0.231 | 0.099 | 0.026 | 0.341 |
| $H_2$ unaccounted (% of produced $H_2$) | 10.9 | 7.6 | 6.9 | 18.3 | 23.0 | 6.67 | 0.227 | 0.059 | 0.389 |

[a, b] Means in the same row with different superscripts differ ($P<0.05$).

[1] Orthogonal contrasts for L = linear and Q = quadratic effects.

**Table 3** *In vitro* dose response effect of nitrate on gas production and composition, and fermentation parameters after 32 h incubation with GLU substrate (100% glucose; experiment 2)

| | Nitrate dose (mM) | | | | | | P-Value[1] | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | 0 | 1 | 2 | 4 | 6 | SED | Dose | L | Q |
| Gas production and composition | | | | | | | | | |
| Total gas production (ml/g) | 350.8$^{a}$ | 339.4$^{a}$ | 323.8$^{ab}$ | 290.5$^{bc}$ | 274.5$^{c}$ | 18.33 | 0.022 | 0.003 | 0.631 |
| $CH_4$ production (ml/g) | 31.0$^{a}$ | 26.2$^{ab}$ | 22.1$^{b}$ | 12.8$^{c}$ | 11.2$^{c}$ | 6.01 | 0.002 | <0.001 | 0.067 |
| $CH_4$ production (% of total gas) | 8.8$^{a}$ | 7.7$^{ab}$ | 6.8$^{b}$ | 4.3$^{c}$ | 4.1$^{c}$ | 1.56 | 0.002 | 0.001 | 0.048 |
| $H_2$ production (ml/g) | 0.29 | 0.20 | 0.34 | 0.35 | 0.22 | 0.153 | 0.809 | 0.948 | 0.531 |
| $H_2$ production (% of total gas) | 0.09 | 0.06 | 0.11 | 0.12 | 0.08 | 0.051 | 0.774 | 0.760 | 0.468 |
| Efficiency of $CH_4$ reduction (%) | -- | 136.7 | 126.0 | 129.2 | 93.5 | -- | -- | -- | -- |
| Fermentation parameters | | | | | | | | | |
| pH | 6.08$^{a}$ | 6.09$^{a}$ | 6.14$^{b}$ | 6.17$^{b}$ | 6.21$^{c}$ | 0.062 | 0.003 | <0.001 | 0.538 |
| $NH_4^+$ (mM) | 9.44$^{a}$ | 10.11$^{a}$ | 12.56$^{ab}$ | 17.14$^{c}$ | 15.09$^{bc}$ | 1.737 | 0.018 | 0.004 | 0.063 |
| Insoluble protein (mg/ml) | 0.26 | 0.20 | 0.22 | 0.22 | 0.17 | 0.053 | 0.641 | 0.276 | 0.923 |
| Total VFA (mmol/g) | 7.08 | 6.92 | 6.84 | 5.30 | 4.82 | 1.168 | 0.155 | 0.036 | 0.826 |
| Acetate (% of total VFA) | 42.9$^{a}$ | 45.3$^{ab}$ | 44.8$^{a}$ | 51.6$^{bc}$ | 57.8$^{c}$ | 2.04 | 0.020 | 0.004 | 0.250 |
| Propionate (% of total VFA) | 42.8$^{a}$ | 41.6$^{ab}$ | 41.5$^{ab}$ | 36.9$^{b}$ | 31.0$^{c}$ | 2.03 | 0.027 | 0.006 | 0.175 |
| Butyrate (% of total VFA) | 13.4 | 11.8 | 13.0 | 11.3 | 11.2 | 1.27 | 0.100 | 0.041 | 0.614 |
| Acetate/propionate | 1.01$^{a}$ | 1.10$^{ab}$ | 1.08$^{a}$ | 1.41$^{b}$ | 1.87$^{c}$ | 0.096 | 0.012 | 0.003 | 0.069 |
| (Acetate+butyrate)/propionate | 1.33$^{a}$ | 1.39$^{ab}$ | 1.40$^{ab}$ | 1.71$^{b}$ | 2.23$^{c}$ | 0.112 | 0.013 | 0.003 | 0.061 |

[a, b] Means in the same row with different superscripts differ (P<0.05).

[1] Orthogonal contrasts for L = linear and Q = quadratic effects.

**Table 4** *In vitro* dose response effect of nitrate on calculated hydrogen production and distribution between fermentation end-products with GLU substrate (100% glucose; experiment 2)

| | Nitrate dose (mM) | | | | | | P-Value[1] | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | 0 | 1 | 2 | 4 | 6 | SED | Dose | L | Q |
| $H_2$ production (mmol/bottle) | | | | | | | | | |
| From acetate | 2.31 | 2.33 | 2.33 | 2.06 | 2.11 | 0.441 | 0.692 | 0.289 | 0.934 |
| From butyrate | 0.72 | 0.63 | 0.68 | 0.47 | 0.42 | 0.152 | 0.085 | 0.021 | 0.996 |
| Total | 3.03 | 2.96 | 3.01 | 2.53 | 2.53 | 0.589 | 0.393 | 0.123 | 0.948 |
| $H_2$ consumption (mmol/bottle) | | | | | | | | | |
| For methane | 2.11[a] | 1.78[ab] | 1.50[b] | 0.87[c] | 0.76[c] | 0.408 | 0.002 | <0.001 | 0.067 |
| For propionate | 1.15 | 1.14 | 1.08 | 0.74 | 0.57 | 0.154 | 0.076 | 0.016 | 0.609 |
| For nitrate reduction | 0.00 | 0.24 | 0.48 | 0.96 | 1.44 | 0.000 | -- | -- | -- |
| For microbial biomass | 0.012 | 0.009 | 0.010 | 0.010 | 0.008 | 0.0021 | 0.604 | 0.276 | 0.958 |
| Total | 3.27 | 3.12 | 3.07 | 2.58 | 2.78 | 0.507 | 0.065 | 0.021 | 0.144 |
| $H_2$ emission (mmol/bottle) | 0.01 | 0.00 | 0.01 | 0.01 | 0.00 | 0.003 | 0.819 | 0.869 | 0.520 |
| $H_2$ unaccounted (mmol/bottle) | -0.24 | -0.15 | -0.07 | -0.06 | -0.25 | 0.265 | 0.902 | 0.986 | 0.408 |
| $H_2$ unaccounted (% of produced $H_2$) | -7.8 | -4.7 | -1.8 | -3.3 | -13.3 | 11.88 | 0.870 | 0.659 | 0.415 |

[a, b] Means in the same row with different superscripts differ (P<0.05).

[1] Orthogonal contrasts for L = linear and Q = quadratic effects.

*Experiment 2*

When glucose was used as substrate, total gas production linearly decreased from 350.8 to 274.5 ml/g as $NO_3^-$ doses increased from 0 to 6 mM $NO_3^-$ (Table 3). Methane production (ml/g and % of total gas) was linearly reduced with $NO_3^-$, ranging from 8.8% of total gas produced in the control treatment to 4.1% of total gas produced for 6 mM $NO_3^-$ (P=0.002). Except for 6 mM $NO_3^-$, the reduction in $CH_4$ emission was higher than the stoichiometrically calculated reduction. Nitrate did not affect gaseous $H_2$ emissions expressed in ml/g or as a percentage of total gas produced.

After 32 h incubation, the pH linearly increased from 6.08 to 6.21 with increasing nitrate concentrations (P=0.003). Nitrate increased $NH_4^+$ concentrations (P=0.018) but did not affect the production of insoluble protein which averaged 0.22 mg/ml. Kinetics of insoluble protein production also confirmed the absence of treatment effect throughout the incubation (Figure 3). Nitrate did not affect total VFA production, but increased the proportion of acetate (P=0.020) while reducing the proportion of propionate (P=0.027). These results led to a linear increase of the ratios acetate/propionate (P=0.012) and (acetate+butyrate)/propionate (P=0.013).

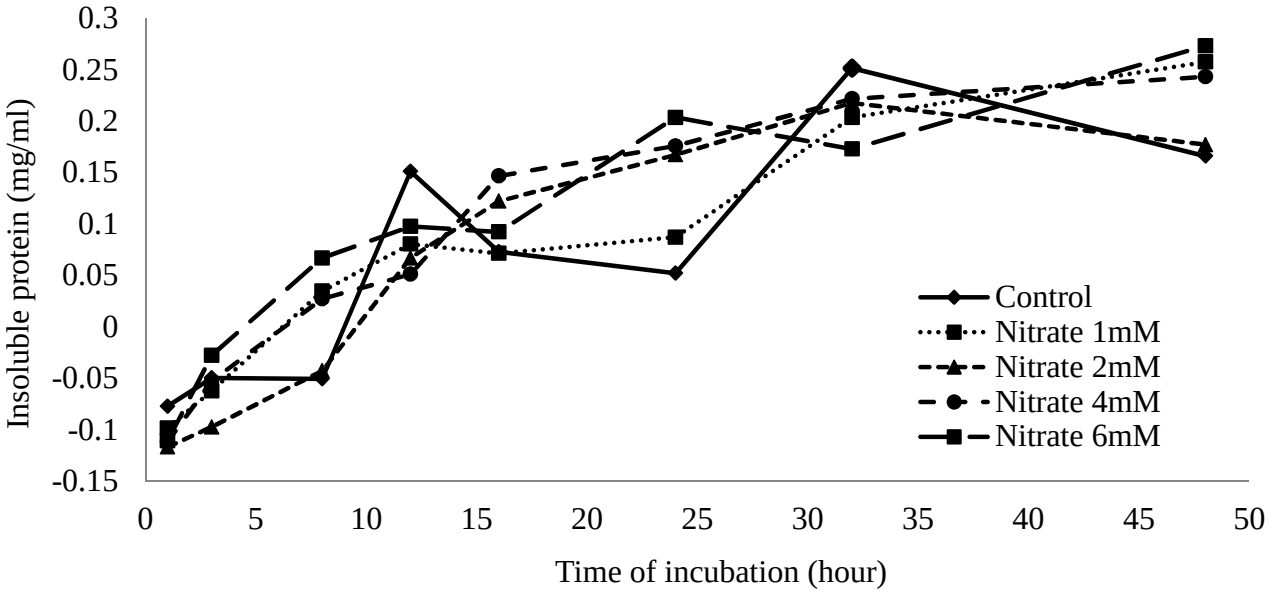


**Figure 3** *In vitro* dose response effect of nitrate on kinetics of insoluble protein during 48 h incubation with GLU substrate (100% glucose; experiment 2)

Treatments did not affect total $H_2$ production which averaged 2.82 mmol/bottle, but total $H_2$ consumption tended to be different between treatments (P=0.065; Table 4). The quantities of

$H_2$ used for microbial biomass were similar between treatments and averaged 0.010 mmol/bottle. Finally, unaccounted $H_2$ was similar and close to zero for all treatments.

## Discussion

*Dose response effect of nitrate on gaseous emissions and rumen fermentation profile*

With 6 mM $NO_3^-$, total gas production was decreased by 26% and 22% with GP and GLU substrates, respectively. Similar observations were made in previous *in vitro* experiments testing similar or higher $NO_3^-$ doses with alfalfa hay (13 mM; Bozic et al., 2009), wheat straw and concentrate (5 and 10 mM; Sakthivel et al., 2012) or alfalfa hay and concentrate (5 and 10 mM; Patra and Zhongtang, 2013; 2014). These results indicate that $NO_3^-$ at a level greater than 5 mM inhibits *in vitro* rumen fermentation. However, $NO_3^-$ did not affect production and composition of VFA with GP substrate. This is in accordance with Patra and Zhongtang (2014), but in contrast to Bozic et al. (2009) where *in vitro* $NO_3^-$ supplementation reduced propionate proportion in total VFA. In the present work, the stability of propionogenesis may be explained by an equilibrium between two opposite actions of nitrate on the $H_2$ pool: i) a reduction of $H_2$ availability for nitrate reduction (electron sink); ii) an increase of $H_2$ availability via its direct toxic effect towards methanogens as indicated by the observed higher gaseous $H_2$ emissions (Janssen, 2010).

In the two *in vitro* experiments of this study, $CH_4$ emissions were linearly reduced with increasing concentrations of $NO_3^-$. The kinetics of $CH_4$ emissions with GP substrate indicated that $NO_3^-$ acts rapidly during the first 10 h. This observation can be related to the quick absorption of $NO_3^-$ by rumen microbes: *in vitro*, microbes used $NO_3^-$ within 10 h incubation in the medium (Shi et al., 2012). *In vivo* on sheep fed 1.3 g $NaNO_3$/kg metabolic weight, the concentration of $NO_3^-$ was decreased by 50% within 5 h postfeeding (Sar et al., 2004). In the rumen of cows fed 3% calcium nitrate, $NO_3^-$ was not even detected 3 h after feeding (Guyader et al., 2014a).

When GP diet was used as a substrate, the observed $CH_4$ inhibition was higher than the stoichiometrically calculated inhibition at levels of $NO_3^-$ exceeding 4 mM, and increased as $NO_3^-$ doses increased. In contrast, when GLU was used, the apparent yield of $CH_4$ reduction was higher than 100%, independent of the $NO_3^-$ concentration. This observation indicated that the assumption that $NO_3^-$ only acts as an electron acceptor is not sufficient enough to understand the $CH_4$-mitigating mechanisms of $NO_3^-$. While the conversion of $NO_3^-$ to $NH_4^+$ requires electrons, a release of gaseous $H_2$ is an indicator for a direct inhibition of

methanogens, and the decrease in gas production indicates a direct inhibitory effect on fermentative microorganisms. As long as $NO_3^-$ is deviating electrons, no $H_2$ emissions will be observed, and only after 10 to 15 h when the $NO_3^-$ is exhausted, gaseous $H_2$ is observed. These results confirm previous *in vitro* (Zhou et al., 2011) and *in vivo* (Van Zijderveld et al., 2010; Guyader et al., 2014c) results showing that $NO_3^-$ have a toxic effect towards methanogens. However, the direct toxicity of $NO_3^-$ against methanogens is dose and substrate dependent, and becomes evident only at concentrations above 4 mM with GP substrate and no such a toxic effect was observed when GLU was used as a substrate.

*Dose response effect of nitrate on $H_2$ metabolic fluxes*

Total $H_2$ production calculated from acetate and butyrate concentrations was similar between treatments, when GP substrate was used. Differences were only observed for $H_2$ consuming pathways such as methanogenesis. For the control treatment, 76% of $H_2$ was directed towards methanogenesis and 13% towards propionate synthesis. These percentages were in the range of previous estimations of $H_2$ distribution between fermentation end-products (Czerkawski, 1986; Mills et al., 2001).

Unaccounted $H_2$ represented between 6.9 and 23.0%, which may be either captured in microbial biomass (Czerkawski, 1986; Mills et al., 2001) or derived from substrates other than glucose on which the calculation is based on (Wolin, 1960). In this balance, we assumed a full transformation of $NO_3^-$ to $NH_4^+$, which is supported by the high efficiencies of $NO_3^-$ reduction. Unaccounted $H_2$ was similar to the control at low level of $NO_3^-$, but higher levels increased the percentage of unaccounted $H_2$. Two hypotheses were tested in order to understand how missing $H_2$ can be used. Firstly, we assumed that formate which production may require $H_2$ via the formate-hydrogen lyase, accumulated in the medium, as shown in previous monoculture of *Ruminococcus flavefaciens* (Wolin et al., 1997). This intermediate of rumen fermentation was also observed *in vitro* when $CH_4$ emissions were inhibited with propynoic acid or ethyl 2-butynoate (Ungerfeld et al., 2006). However, although in the present incubation formate was not determined, no formate was found in response to 2 and 8 mM $NO_3^-$ in separate *in vitro* incubations with GP substrate (data not shown).

Assuming that microbes require 0.41 moles $H_2$ per kg microbes (Mills et al., 2001), it was expected that microbial biomass was increased with high doses of $NO_3^-$, using a part of unaccounted $H_2$. However, treatments did not affect insoluble protein concentrations, showing that $NO_3^-$ did not enhance microbial synthesis. This result confirms previous *in vitro* experiments reporting an absence of $NO_3^-$ (5 and 10 mM doses) effect on bacterial and

protozoal concentrations (Zhou et al., 2011; Patra and Zhongtang, 2013; 2014). This is also in accordance with an *in vivo* experiment reporting no difference in microbial protein supply based on urinary excretion of purine derivatives of non-lactating cows supplemented with $NO_3^-$ (2.3% in DM; Guyader et al., 2014a). In addition, the estimated percentage of $H_2$ directed towards microbial biomass was very low and ranged between 0.3 and 0.3%, confirming the low contribution of microbes to $H_2$ consumption (Mills et al., 2001).

**Conclusions**

Nitrate is an efficient $CH_4$-mitigating strategy but it can be used only to a limited extent, before fermentation is negatively affected. The *in vitro* threshold appears to be between 2 to 4 mM, which would correspond to a supplementation to animals situated between 1.2 and 2.5% of DM. The difference between observed and theoretical $CH_4$ production shows that this additive acts as an electron acceptor, but its mechanisms of action must also involve a direct toxic effect on methanogens. In addition, the study of $H_2$ distribution between fermentation end-products shows that $NO_3^-$ must enhance another unknown $H_2$ consuming pathway, different from $H_2$ emitted or captured for $NO_3^-$ reduction, and for production of $CH_4$, VFA or microbial biomass.

**Conflict of interest**

None.

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# General discussion

Methane released by ruminants is the main greenhouse gas at the farm level (Veysset et al., 2010) and constitutes an energetic loss for the animal, ranging from 2 to 12% of its GEI (Johnson and Johnson, 1995). Therefore, this PhD thesis takes part in the global context of $CH_4$ mitigation, in order to reduce the negative environmental impacts of ruminants while improving their feed efficiency.

Knowing that $H_2$ is the limiting substrate of methanogenesis in the rumen, the objective of this PhD thesis consisted in studying the importance of the different $H_2$ metabolic pathways (production and consumption), in order to determine the more efficient way to manipulate $H_2$ pool in the rumen. The final purpose of this work consists in proposing new dietary $CH_4$-mitigating strategies. We assumed that acting on both reduction of $H_2$ production and stimulation of $H_2$ consumption by a competitive pathway to methanogenesis decreases $CH_4$ production to a higher extent than when acting on a single pathway.

Our scientific approach was divided in two parts. Firstly, the bibliographical approach detailed the biological and thermodynamic mechanisms of $H_2$ production and utilization in the rumen *via* a classic literature review. In addition, a meta-analysis reported the relationship between rumen protozoa and $CH_4$ emissions. Secondly, the experimental approach assessed the effect of association of dietary strategies on $CH_4$ emissions of non-lactating and lactating cows. The originality of our work consisted in associating dietary treatments with different mechanisms of action on $H_2$ pool (reducing $H_2$ production or consuming $H_2$). Moreover, the distribution of $H_2$ between fermentation end-products was estimated *in vitro* with a strategy acting on $H_2$ utilization.

In the following discussion, we will focus on the main original results obtained during this PhD thesis. This section will be divided into three parts:

1/ we will give an experience feedback on new equipment acquired during this PhD thesis ($CH_4$-open chambers and $H_2$-sensors), and we will assess precision and accuracy of $CH_4$ emissions and rumen dissolved $H_2$ concentrations obtained in cows fed control diets.

2/ we will assess the relevance of the tested $CH_4$-mitigating strategies on methanogenesis, but also on overall digestive and zootechnical performances. Rumen fermentation mechanisms of these $CH_4$-mitigating strategies will be highlighted, by relating them with distribution of $H_2$ in the different fermentation end-products and with modification in the microbiota.

3/ we will discuss the possibility of a practical use at the farm scale of the most efficient $CH_4$-mitigating dietary strategy tested in this PhD thesis.

# I. PRECISION AND ACCURACY OF METHANE EMISSIONS AND RUMINAL DISSOLVED HYDROGEN CONCENTRATIONS IN COWS FED CONTROL DIETS

During this PhD thesis, two new methods were implemented in the team to monitor cows' individual kinetics of i) enteric $CH_4$ emissions using open chambers and ii) rumen dissolved $H_2$ concentrations using $H_2$-sensors. In the following section, we will give an experience feedback on these two devices and we will assess the precision and accuracy of our data obtained on cows fed control diets by comparing them with the literature.

## *1.1. Precision and accuracy of methane emissions*

**Table 11** Compiled data of methane emissions obtained in the experiments of this PhD thesis with non-lactating and lactating cows

| Experiment | Experimental design | | | | Methane emissions (± SD) | | |
|---|---|---|---|---|---|---|---|
| | Animal (n) | Forages (% of DM) | Methane measurement technique | Days in chambers | g/day | g/kg DMI | % of GEI |
| 1 & 3 | Non-lactating cows (8) | Grass hay (50) | Open chambers | 4 | 310.5 (± 16.50) | 25.2 (± 1.56) | 7.2 (± 0.45) |
| 2 & 3 | Lactating cows (15) | Corn silage, grass hay (60) | Open chambers | 4 or 2 | 450.9 (± 111.77) | 21.2 (± 3.50) | 6.0 (± 0.99) |

n: number of animals; SD: Standard deviation

In this PhD thesis (Table 11), 8 non-lactating cows (experiments 1 and 3) were fed a same control hay-based diet in restricted conditions (90% of *ad libitum* intake). Their $CH_4$ emissions were measured in open chambers for 4 consecutive days. Fifteen lactating cows (experiments 2 and 3) were fed *ad libitum* a same control corn silage-based diet. During measurement of their $CH_4$ emissions in open chambers for 4 or 2 days, animals were restricted fed (95% of *ad libitum* intake).

### 1.1.1. Experience feedback on open chambers for cattle

Our open chambers allowed to measure daily kinetics of enteric $CH_4$ emissions in cattle (see experiment 1 for a detailed description of chambers). Chambers were designed to be spacious and comfortable for the animals (4-cm thick mattress, 2.5-m² lying area) in order to avoid disturbance of cows' behavior and performances during their stay inside. In addition, animals were used to be attached in the barn before to be moved in open chambers. The levels of DMI in chambers averaged 12.4 (day-to-day coefficient of variation, CV = 1.3%) for non-lactating and 21.2 (day-to-day CV = 2.0%) kg/day for lactating cows, and were similar to the levels of DMI measured the week preceding or following $CH_4$ measurement (12.4 kg/day, day-to-day CV = 1.1% for non-lactating cows; 20.8 kg/day, day-to-day CV = 3.6% for lactating cows). The constant DMI and milk production of cows between inside and outside chambers reflected that animals easily adapted to open chambers and that our experimental conditions are good enough to measure accurate $CH_4$ emissions in cows.

In our experimental conditions, the day-to-day variability of $CH_4$ emissions (g/day) within animals was low and similar between non-lactating and lactating cows (4.1%, on average). This variability level was comparable with data reported in the literature, which ranged between 4.3 and 7.2% for animals placed in respiration chambers for a minimum of 3 consecutive days (dairy and beef cattle, n = 87, Blaxter and Clapperton, 1965; dairy cattle, n = 16, Grainger et al., 2007).

Variability of $CH_4$ emissions (g/day) between animals averaged 5.3% for non-lactating cows fed a hay-based diet and 24.8% for lactating cows fed a corn silage-based diet. These levels remained comparable with reviews cited previously (8.1%, no indication about diets, Blaxter and Clapperton, 1965; 17.8%, 75% forage in diet, Grainger et al., 2007). The higher variability of $CH_4$ emissions between lactating cows is consistent with their higher DMI variability (18.6% for lactating cows fed sub *ad libitum versus* 7.9% for dry cows restricted fed). Then, we also confirmed a previous study reporting that the CV between animals is larger when intake is not restricted (Grainger et al., 2007).

**Table 12** Equations used to estimate $CH_4$ emissions of cows fed control diets in all experiments of this PhD thesis

| Reference | n | Animals | Methane measurement technique | Forage (% of DM) | DMI%BW [min-max] | $CH_4$ (g/day) [min-max] | Equation[1] | RMSE (unit) | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|
| Sauvant et al., 2011 **[1]** | 976 | Dairy and beef cattle, sheep and goat[2] | Chambers (n=976) | 60 | 1.61 [0.56-4.01] | NA[4] | $CH_4$ (g/day) = (7.14 + 0.22 × DOM) / DMI | 2.70 (g/kg DMI) | 0.81 |
| Mills et al., 2003 **[2]** | 159 | Dairy cattle (n=159) | Chambers (n=159) | 55 | NA[3] | 479.5 [325.0-605.9] | $CH_4$ (g/day) = (5.93 + 0.92 × DMI) × Z | 1.82 (MJ/day) | 0.60 |
| Ellis et al., 2007 **[3]** | 172 | Dairy (n=89) and beef cattle (n=83) | Chambers (n=101) SF6 (n=42) Others (n=29) | 75 | 2.25 [NA] | 236.8 [56.4-499.6] | $CH_4$ (g/day) = (3.27 + 0.74 × DMI) × Z | 0.28 (MJ/day) | 0.68 |
| Ellis et al., 2007 **[4]** | 83 | Beef cattle (n=83) | Chambers (n=44) SF6 (n=37) Others (n=2) | 80 | 1.99 [NA] | 183.2 [56.4-345.1] | $CH_4$ (g/day) = (3.96 + 0.561 × DMI) × Z | 0.26 (MJ/day) | 0.44 |
| Ellis et al., 2007 **[5]** | 89 | Dairy cattle (n=89) | Chambers (n=57) SF6 (n=5) Others (n=27) | 70 | 2.37 [NA] | 286.9 [86.5-499.6] | $CH_4$ (g/day) = (3.23 + 0.809 × DMI) × Z | 0.26 (MJ/day) | 0.65 |
| Ramin and Huhtanen, 2013 **[6]** | 207 | Dairy (n=145) and beef (n=62) cattle | Chambers (n=207) | 70 | 2.18 [0.78-5.23] | 218.7 [9.2-541.7] | $CH_4$ (g/day) = (20 + 35.8 × DMI − 0.50 × $DMI^2$) × 0.71427 | NA | -- |
| Sauvant and Nozière, 2013 **[7]** | 450 | Dairy and beef cattle, sheep and goat[2] | Chambers (n=450) | 60 | 1.61 [0.56-4.01] | NA[4] | $CH_4$ (g/day) = (45.42 – 6.66 × (DMI:BW) + 0.75 × $(DMI:BW)^2$ + 19.65 × PC – 35.0 × $PC^2$ - 2.69 × (DMI:BW) × PC) × DOMI | 2.3 (g/kg DOM) | -- |

n: number of treatments; RMSE: residual mean square error; NA: non-available

[1] Z = conversion factor between $CH_4$ expressed in MJ/day to $CH_4$ expressed in g/day = 20.0638; DOM (% of DM) = digestible OM in diet = OM content of the diet (% of DM) × OM digestibility (0-1); DMI (kg/day) = dry matter intake; PC = concentrate proportion (0-1); DOMI = digestible OM intake (kg/day) = DOM × DMI

[2] Proportions not available

[3] DMI = 19.6 kg/day, with minimum and maximum: 12.5 and 28.4 kg/day

[4] $CH_4$ = 18.3 g/kg DMI, with minimum and maximum: 13.6 and 23.0 g/kg DMI

### 1.1.2. Comparison of methane emissions with the literature

*Daily pattern of $CH_4$ emissions.* In the experiments of this PhD thesis, animals were fed twice daily and the daily patterns of $CH_4$ emissions were similar between cows fed the control diets. Methane emissions increased quickly following feed intake to reach a peak 2 h after feeding, and then decreased progressively until the next feeding. These daily patterns of methanogenesis according to feeding frequency are in accordance with previous observations (Grainger et al., 2007; Janssen, 2010; Van Zijderveld et al., 2010).

*Difference between non-lactating and lactating cows.* Expressed in g/day, $CH_4$ emissions of non-lactating cows fed a hay-based diet were lower than lactating cows fed a corn silage-based diet (310.5 *versus* 450.9 g/day). This expected result is explained by the lower intake level of non-lactating cows compared to lactating cows (12.4 *versus* 21.2 kg DMI/day). Indeed, the positive correlation between $CH_4$ emissions (g/day) and DMI is well known (Reynolds et al., 2011; Ramin and Huhtanen, 2013).

Inversely, when expressed in g/kg DMI or as a percentage of GEI, $CH_4$ emissions of non-lactating cows (hay-based diet) were higher than lactating cows (corn silage-based diet; 25.2 *versus* 21.2 g/kg DMI; 7.2 *versus* 6.0% GEI). This difference may be explained by two confounded effects. The first one is related to the higher intake level of lactating cows compared to non-lactating cows, which decreased the feed retention time in the rumen, lowering the time for microbial fermentation of feed substrates (Reynolds et al., 2011). The second one is related to the forage nature of the basal diet. Forage preservation may affect enteric $CH_4$ production which tends to be lower when forages are ensiled than when they are dried (Martin et al., 2010). From direct comparisons, Doreau et al. (2011) also reported that lactating cows fed silage-based diets produce less $CH_4$ (g/per kg milk) than those fed hay-based diets.

*Comparison of observed and predicted $CH_4$ emissions*. In order to assess the coherence of our $CH_4$ emissions, data from individual cows fed control diets were confronted to $CH_4$ emissions estimated with equations from the literature. To predict enteric $CH_4$ emissions, several equations are available in the literature, which are based on various criteria such as intake level, diet composition, production level of animals or rumen fermentation parameters. In the present work, we selected 7 predictive equations (Table 12) for the following reasons:

1/ They predict $CH_4$ emissions from the level of DMI (Ellis et al., 2007; Mills et al., 2003; Ramin and Huhtanen, 2013) which is the main determinant of $CH_4$ production (Reynolds et al., 2011; Ramin and Huhtanen, 2013), and/or from the level of digestible OM (DOM) in the diet (Sauvant et al., 2011; Sauvant and Nozière, 2013) which is a good predictor of $CH_4$ emissions, as it is statistically related with the level of fermented OM in the rumen (Sauvant et al., 2011).
2/ They were developed from large database built with *in vivo* data collected from animals fed diets containing proportions of concentrate (20-50%) overlying those of our experimental diets (40-50% concentrate).
3/ They were developed from data of $CH_4$ emissions mostly measured with chambers, as realized in this work (93% with chambers *versus* 4% with SF6 *versus* 3% with other techniques).

We adopted two approaches for comparison of observed and predicted $CH_4$ emissions (g/day). To compare **absolute values**, a T-test was applied between observed and predicted $CH_4$ emissions for each equation. To check **variations in $CH_4$ emissions**, the relationship between observed and predicted $CH_4$ emissions was tested for each equation using the general linear model (GLM) procedure:

$$\text{Observed } CH_4 = \alpha + \beta \times \text{predicted } CH_4$$

Where $\alpha$ = the overall intercept and $\beta$ = the overall slope. Non-significant intercepts were considered as equal to 0. Slopes were compared to 1 by calculating T ($T = (\text{slope} - 1)/SD_{slope}$), which was compared to $t_\alpha$ obtained from the T-Student table ($\alpha = 0.05$). If $T > t_\alpha$, the slope was considered different from 1. Statistical analyses were performed with Minitab (Version 16).

When considering all data from lactating or non-lactating cows fed control diets (Table 13; Figure 20), we showed that absolute data between observed and predicted $CH_4$ emissions were positively correlated (average $R^2$ = 76.6% on average) whatever the equation ($P<0.001$). Absolute $CH_4$ emissions between observed and predicted were similar ($P>0.05$) with equations 2, 5, 6 and 7, whereas observed $CH_4$ emissions were significantly higher than predicted with equations 1, 3 and 4 (average bias = +142.5, +68.0 and +119.1 g/day, respectively; $P<0.05$). Concerning variations between observed and predicted $CH_4$ emissions, all intercepts tended or were equal to 0, and slopes of regressions were significantly equal to 1 ($P<0.05$) for all equations, except for equations 4 and 6. The differences between observed and predicted $CH_4$ emissions with equations 1, 3 and 4 may be explained by i) the different animal species and type of cattle production (equation 1 is proposed for sheep and cattle,

equation 4 is proposed for beef cattle), and ii) the higher concentrate proportion in our experiments (40-50%) compared with the average concentrate proportion in the dataset used to generate the equations 3 and 4 (20-25%).

**Table 13** Comparison and relationship between observed and predicted $CH_4$ emissions (g/day) of lactating and non-lactating cows (n=23) used in the experiments of this PhD thesis.

| Equation | Predicted $CH_4$ (±SD)[1] | *P-Value* (T-test) | Intercept (SE) | Slope (SE) | Slope = 1 | $R^2$ | RMSE |
|---|---|---|---|---|---|---|---|
| [1] | 259.6 (±78.4) | <0.001 | 74.2$^{\dagger}$ (40.70) | 1.26$^{***}$ (0.150) | **Yes** | 0.77 | 55.3 |
| **[2]** | 452.8 (±98.7) | **0.112** | -53.3$^{ns}$ (54.83) | 1.01$^{***}$ (0.118) | **Yes** | 0.77 | 54.8 |
| [3] | 334.1 (±79.4) | 0.023 | -15.7$^{ns}$ (50.50) | 1.25$^{***}$ (0.147) | **Yes** | 0.77 | 54.8 |
| [4] | 283.0 (±60.2) | <0.001 | -64.7$^{ns}$ (56.14) | 1.65$^{***}$ (0.194) | No | 0.77 | 54.8 |
| **[5]** | 358.3 (±86.8) | **0.148** | -7.8$^{ns}$ (49.60) | 1.14$^{***}$ (0.135) | **Yes** | 0.77 | 54.8 |
| [6] | 350.2 (±65.6) | **0.064** | -116.8$^{\dagger}$ (67.56) | 1.48$^{***}$ (0.190) | No | 0.74 | 58.4 |
| **[7]** | 362.4 (±90.6) | **0.195** | 6.7$^{ns}$ (48.68) | 1.09$^{***}$ (0.131) | **Yes** | 0.77 | 55.5 |

SE: Standard error

[1] Observed $CH_4$ emissions averaged 402 ± 112.8 g/day.

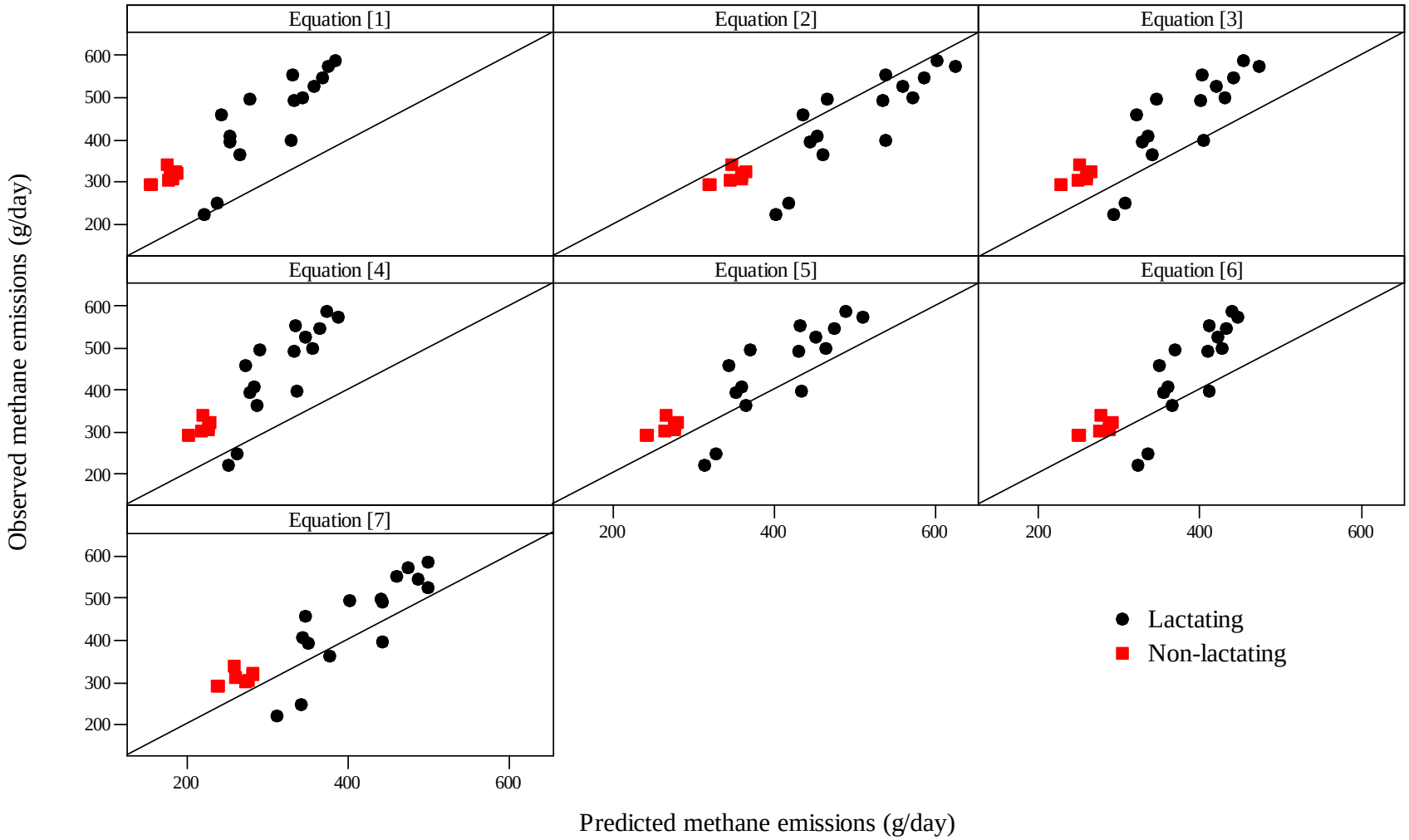


**Figure 20** Relationship between observed and predicted methane production (g/day) of lactating (n=15) and non-lactating (n=8) cows fed control diets in the different experiments of this PhD thesis

## *1.2. Precision and accuracy of ruminal dissolved hydrogen concentrations*

This PhD thesis is the first work reporting kinetics of dissolved $H_2$ concentrations in the rumen of fistulated cows using indwelling $H_2$-sensors (experiment 1).

### 1.2.1. Experience feedback on $H_2$-sensors

We succeeded to adapt an $H_2$-sensor (commonly used in marine research) for continuous and *in situ* measurement of dissolved $H_2$ concentration in the rumen. This system counteracted the main disadvantages of previous $H_2$-measurement devices (detailed in Materials and methods section of this manuscript): i) it measured kinetics of dissolved $H_2$ concentrations, ii) it detected quick modification of $H_2$ concentrations (90% response in 15 sec) which was important knowing that the turnover time of $H_2$ in the rumen is ~0.08 sec, and iii) it had a low limit of $H_2$ quantification (0.3 µ*M*).

From a practical point of view, the full system did not require important equipment, and the sensor size was rather small which did not disturb the ruminal environment. However, the glass-made tip of the sensor was very fragile, and the sensor required a strong home-made protection prior to its insertion into the rumen, and this was very challenging if the animal just ate. To counteract this issue, we inserted the sensor before morning feeding and removed it before afternoon feeding. Kinetics of dissolved $H_2$ concentrations were measured for 5 h postfeeding.

As we only had one available and functional $H_2$-sensor, measurements were carried out only one day per cow per experimental period. Then, for the whole experiment, we collected 4 daily kinetics obtained on 4 cows fed the control diet. For this reason, variability between days was impossible to estimate for this measure.

Rumen dissolved $H_2$ concentrations of the 4 non-lactating cows fed a same control diet presented an important inter-animal variability. Figure 21 shows that the highest variability levels were observed during the time outside feeding time (from 2.5 to 3 h after feeding). This result highlights the importance of repeating the measurement for several days for a same animal. In our case, this was impossible because of limitation in $H_2$-sensors availability.

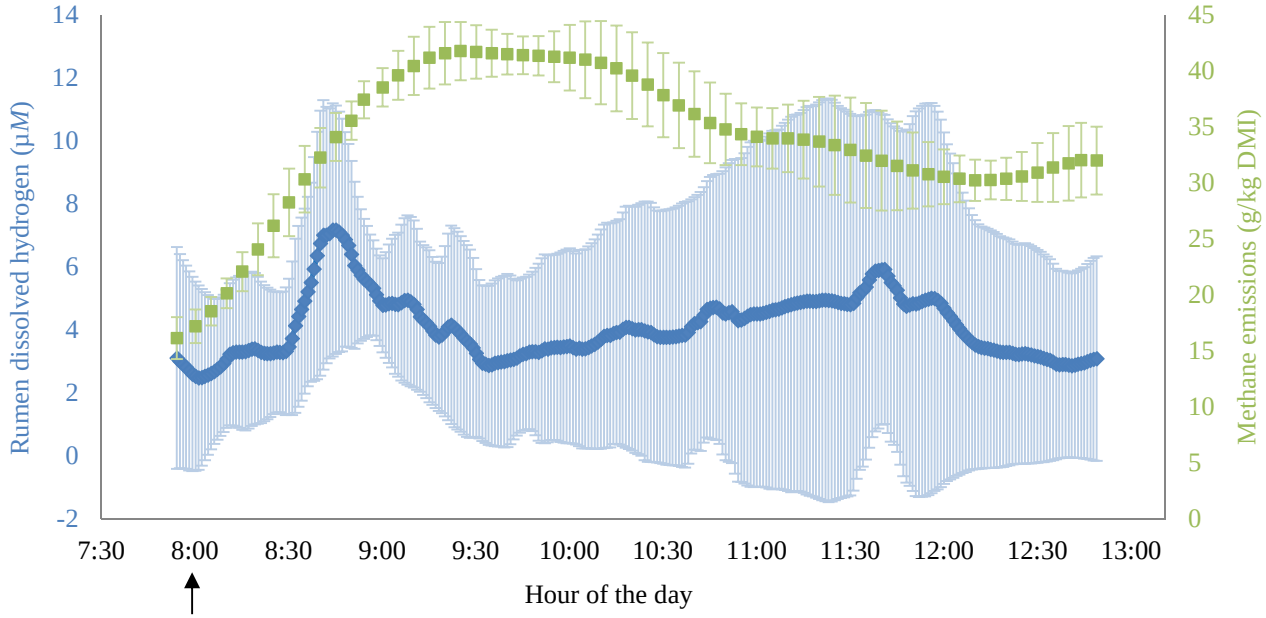


**Figure 21** Average rumen dissolved hydrogen concentrations and methane emissions up to 5 h after feeding a similar hay-based diet to four non-lactating cows. Errors bars indicate SD. The arrow indicates time of feeding.

1.2.2. Comparison of ruminal dissolved hydrogen concentrations with the literature

During the 5 h after feeding, dissolved $H_2$ concentration in the rumen of cows fed a hay-based diet averaged 4.1 µ*M* with an interval situated between 2.5 and 7.2 µ*M*. These concentrations were low, but in the range of previous observations (0.1-50 µ*M*) given by Janssen (2010).

The maximum ruminal dissolved $H_2$ concentrations (7.2 µ*M*) was observed less than 1 h after feeding. This postprandial peak of rumen dissolved $H_2$ concentration was situated upstream of the observed postprandial peak of $CH_4$ emissions, coinciding with previous observations (Swainson et al., 2011). This postfeeding $H_2$ peak probably corresponded to the release of $H_2$ coming from fermentation of fresh feed ingested. This pattern was in accordance with previous studies on cattle or sheep, which also observed a rise of ruminal dissolved $H_2$ concentrations between 10 min and 3 h after feeding (Morgavi et al., 2012; Robinson et al., 1981; Smolenski and Robinson, 1988). As for $CH_4$ emissions, we assume that the extent and time of the postfeeding $H_2$ peak is dependent on the fermentation rate of diet components and on the feeding frequency.

**The aim of this PhD thesis was to propose new dietary strategies to mitigate $CH_4$ emissions in ruminants *via* a modification of $H_2$ availability in the rumen. Quantification of individual $CH_4$ emissions was an essential measurement in this work, as well as**

**dissolved $H_2$ concentrations in the rumen as indicator of $H_2$ availability. Monitoring kinetics of these two parameters allowed getting a better insight of mechanisms involved in $CH_4$ mitigation. Consequently, four open $CH_4$ chambers for cattle were implemented in the team and $H_2$-sensors were adapted to the rumen environment. Overall results indicate that in our experimental conditions, kinetics of $CH_4$ emissions were precise and accurate. Data on $H_2$ kinetics were original, but additional research is required to assess the reproducibility and repeatability of measurements. In conclusion, we confirm that these two devices were adapted to evaluate the efficiency and understand the mechanisms of actions of the selected dietary $CH_4$-mitigating strategies.**

**Table 14** Compiled data from the literature on the effects of lipids from linseed supplementation to cattle or sheep on methane emissions, total tract digestibility and nitrogen balance

| Reference | Animal species | Forages (% of DM) | Linseed form | Added fat[1] (% of DM) | Methane reduction (g/kg DMI, % per 1% added C18:3) | Effect on total tract digestibility | Effect on nitrogen balance |
|---|---|---|---|---|---|---|---|
| Experiment 1, this PhD thesis | Dry cows | Grass hay (50) | Linseed oil | 2.6 | -6.6 | No effect | No effect |
| Machmüller et al., 2000 | Lambs | Corn silage, grass hay (76) | Crude linseed | 2.4 | -6.0 | No effect | NA |
| Martin et al., 2008 | Dairy cows | Corn silage, grass hay (65) | Crude linseed<br>Extruded linseed<br>Linseed oil | 4.2<br>4.4<br>5.8 | -2.5<br>-6.0<br>-9.0 | dOM[3]: -4%<br>dNDF[3]: -7%<br>dADF[3]: -6% | NA |
| Chung et al., 2011 | Dry cows | Grass hay (48) | Crude linseed | 5.6 | -0.91 | dOM: -3%<br>dNDF: -7%<br>dADF: -18% | NA |
| | Dry cows | Barley silage (48) | Crude linseed | 4.8 | -6.8 | dOM: -7%<br>dNDF: -20%<br>dADF: -28% | NA |
| Martin et al., 2011 | Dairy cows | Grass silage/hay (57) | Extruded linseed | 3.0 | -4.7 | NA | NA |
| | Dairy cows | Pasture (79) | Extruded linseed | 2.0 | -8.1 | NA | NA |
| Veneman et al., 2014 | Dairy cows | Grass/maize silage (NA) | Linseed oil | 2.6[2] | -0.59 | NA | NA |
| | Dairy cows | Corn silage (NA) | Linseed oil | 2.6[2] | +0.4 | NA | NA |
| **AVERAGE** | | | | | **-4.6** | | |

NA: Data not available

[1] Based on ether extract content of the diet

[2] Values based on estimation, knowing that in our experiment, 4% linseed oil = 2.6% added fat

[3] Extent of reduction in total tract digestibility similar between treatments

**Table 15** Compiled data from the literature on the effects of tea saponin (*C. sinensis* or *assamica*) supplementation to cattle or sheep on methane emissions, total tract digestibility and nitrogen balance

| Reference | Animal species | Forages (% of DM) | Distribution method of tea saponin powder | Tea saponin (% of DM) (% of active saponin compound ) | Methane reduction (g/kg DMI, % per 1% added saponin) | Effect on total tract digestibility | Effect on nitrogen balance |
|---|---|---|---|---|---|---|---|
| Experiment 3, this PhD thesis | Dry cows | Grass hay (50) | In a pellet | 0.77 (0.52) | -4.0 | No effect | No effect |
| | Dairy cows | Corn silage, grass hay (60) | In a pellet | 0.76 (0.52) | +17.9 | dADF: +8% | No effect |
| Yuan et al., 2007 | Adult sheep | Lucerne hay (60) | Mixed with feed | 0.5 (NA) | -17.4 | NA | NA |
| Mao et al., 2010 | Lambs | Chinese wild rye (60) | Mixed with feed | 0.4 (NA) | -68.7 | NA | NA |
| Zhou et al., 2011 | Adult sheep | Chinese wild rye (60) | Mixed with feed | 0.4 (0.24) | -26.5[1] | NA | NA |
| Li and Powers, 2012 | Steers | Corn silage (46) | Mixed with feed | 0.25 (0.06)<br>0.50 (0.12) | -29.2<br>-1.0 | NA | No effect |
| **AVERAGE** | | | | | **-26.4** | | |

NA: Data not available

[1] DMI was not available. We assumed that DMI was similar to Mao et al. (2010)

**Table 16** Compiled data from the literature on the effects of nitrate supplementation to cattle or sheep on methane emissions, total tract digestibility and nitrogen balance

| Reference | Animal species | Forages (% of DM) | Nitrate source | Nitrate dose (% of DM) | Methane reduction (g/kg DMI, % per 1% added nitrate) | Effect on total tract digestibility | Effect on nitrogen balance |
|---|---|---|---|---|---|---|---|
| Experiment 1, this PhD thesis | Dry cows | Grass hay (50) | Calcium nitrate | 2.3 | -9.6 | No effect | No effect |
| Experiment 3, this PhD thesis | Dry cows | Grass hay (50) | Calcium nitrate | 2.3 | -11.7 | No effect | No effect |
| Nolan et al., 2010 | Adult sheep | Oaten hay (100) | Potassium nitrate | 2.5 | -9.5 | No effect | NA |
| Van Zijderveld et al., 2010 | Adult sheep | Corn silage, barley straw (90) | Calcium nitrate | 2.6 | -12.2 | NA | NA |
| Van Zijderveld et al., 2011 | Dairy cows | Corn silage, dried alfalfa, barley straw (66) | Calcium nitrate | 2.1 | -7.9 | No effect | No effect |
| Hulshof et al., 2012 | Steers | Sugar cane (60) | Calcium nitrate | 2.2 | -12.3 | NA | NA |
| Li et al., 2012 | Lambs | NA | Calcium nitrate | 2.3 | -15.4 | No effect | No effect |
| El-Zaiat et al., 2014 | Lambs | Grass hay (60) | Calcium nitrate | 3.4 | -9.7 | NA | NA |
| Lee et al., 2014a | Steers | Forage (55) | Calcium nitrate | 2.3 | -8.0 | NA | No effect |
| de Raphélis-Soissan et al., 2014 | Adult sheep | Oaten hay (100) | Calcium nitrate | 2.0 | -7.5 | NA | NA |
| Lund et al., 2014 | Dairy cows | Grass/clover/corn silage (58) | Calcium nitrate | 2.0 | -12.5 | NA | NA |
| Veneman et al., 2014 | Dairy cows | Grass/corn silage (NA) | Calcium nitrate | 2.0 | -6.8 | NA | NA |
| | Dairy cows | Corn silage (NA) | Calcium nitrate | 2.0 | -8.2 | NA | NA |
| **AVERAGE** | | | | | **-10.1** | | |

NA: Data not available

# II. OVERALL EFFECT OF DIETARY STRATEGIES ON METHANE EMISSIONS AND COWS' PERFORMANCES

## *2.1. Additive methane-mitigating effect of strategies acting on hydrogen production and consumption: validation of our initial hypothesis*

The purpose of this PhD thesis consisted in proposing new efficient dietary $CH_4$-mitigating strategies acting on $H_2$ availability for methanogens. We assumed that decreasing $H_2$ production AND stimulating $H_2$ consumption by a competitive pathway to methanogenesis, reduce $CH_4$ production to a higher extent than when acting on a single pathway.

To reduce methanogenesis *via* a reduction of $H_2$ production, we chose to test lipids from linseed and tea saponin. Indeed, our meta-analysis (Guyader et al., 2014) and previous reviews (Beauchemin et al., 2008; Doreau et al., 2011; Gerber et al., 2013a) highlighted that lipids and plant extracts would have a toxic effect towards protozoa, which are the main $H_2$-producers in the rumen. For lipids, we focused on PUFA from linseed (linolenic acid, C18:3), which have been reported as the most efficient PUFA to mitigate $CH_4$ (Doreau et al., 2011). In addition, linseed supplemented to ruminants has nutritional benefits by improving milk and meat fatty acids profiles (Chilliard et al., 2009; Scollan et al., 2001). Concerning plant extracts, we selected tea saponin, as it would be the most promising saponin source reducing $CH_4$ among the large family of plant extracts (Gerber et al., 2013a; Wang et al., 2012). Moreover, an *in vitro* experiment showed its positive effect on OM digestibility (Wei et al., 2012).

To reduce $H_2$ availability for methanogenesis, the other strategy consisted in supplementing animals with additives consuming $H_2$ (instead of methanogens) and without affecting protozoa. In our literature review, we reported that nitrate may act as a $H_2$-sink in the rumen, and recent reviews showed that all published experiments using this additive resulted in $CH_4$ mitigation (Doreau et al., 2014a; Lee and Beauchemin, 2014b).

The doses of linseed, tea saponin and calcium nitrate used in the present work were determined in order to reach a 15-20% $CH_4$ reduction when these treatments were fed individually (Doreau et al., 2011; Lee and Beauchemin, 2014a; Mao et al., 2010). Assuming an additive effect on $H_2$ availability in the rumen, their association (nitrate plus linseed and nitrate plus tea saponin) was expected to reduce $CH_4$ emissions by 30-40%.

To be adopted by farmers and consumers, a feeding strategy reducing $CH_4$ emissions must do so, without adverse effects on animals' digestive efficiency, performances, quality of products and health. For these reasons, the overall effect of the different selected dietary strategies was assessed by considering not only methanogenesis but also all the parameters cited above. For linseed and tea saponin, we closely monitored their effect on diet digestibility, knowing that more than 5% added fat may reduce *in vivo* total tract digestibility of diets (Martin et al., 2010; Martin et al., 2008), and that tea saponin may improve *in vitro* nutrients digestibility (Wei et al., 2012). In addition, having in mind that nitrate is a N source with a potential toxicity for animals (methemoglobinemia; Lee et al., 2014b) and human health (nitrate and nitrite accumulation in animals products), we also carefully assessed nitrate effect on N release, animals' health and the concentration of N-derivatives compounds in milk.

### 2.1.1. Effect of linseed fed individually to reduce hydrogen production in the rumen on methane emissions and overall cows' performances

Lipids from linseed (4% linseed oil in DM corresponding to 2.6% added fat) fed individually to non-lactating cows (n = 4 in experiment 1) did not affect intake, total tract diets digestibility and N balance. These results were in accordance with the literature, reporting that less than 4% added fat in a diet does not alter animals' intake, digestive processes and performances (Table 14). However, Martin et al. (2011) observed a reduction of DMI (-7%) without effect on milk yield of lactating cows fed grass silage supplemented with extruded linseed (3% added fat in DM).

Daily pattern of $CH_4$ emissions indicated that linseed acted all along the day (Figure 3, experiment 1). Its supplementation decreased daily $CH_4$ emissions (g/kg DMI) by 17.2% on average, corresponding to a $CH_4$ reduction of 6.6% per percent added fat in the diet. This result was in accordance with the majority of previous *in vivo* studies (Table 14) and with a meta-analysis reporting that 1% additional linolenic acid in the diet induces a 5.6% $CH_4$ reduction (Doreau et al., 2011).

The $CH_4$-mitigating effect of lipids is not systematic (Chung et al., 2011; Veneman et al., 2014). The extent of $CH_4$ decrease with lipids is proportional to the level and availability of lipids supply (Martin et al, 2010; Doreau et al., 2011), but these two factors did not explain data of Chung et al., (2011) and Veneman et al., (2014). In the trial of Veneman et al. (2014), lactating cows from New Zealand were used. We assumed that the $CH_4$-mitigating effect of linseed is dependent on the rumen microbiota, which is related to animals' environmental

growing and living conditions. This hypothesis was confirmed in an *in vitro* experiment, using rumen inoculum from NZ cows fed pasture, in which we reported that linseed oil used in our *in vivo* experiment (experiment 1) did not modify *in vitro* $CH_4$ production or rumen fermentation parameters (Muetzel et al., unpublished data). This supports the interest of current international programs such as the Global Rumen Census, which compare the diversity of microbial communities from rumen samples taken on a large diversity of ruminants throughout the world.

**In conclusion, we confirmed that linseed oil supplementation to cattle (2.6% added fat in DM) is an efficient $CH_4$-mitigating strategy without reducing digestive efficiency in cows.**

### 2.1.2. Effect of tea saponin fed individually to reduce hydrogen production in the rumen on methane emissions and overall cows' performances

Tea saponin (0.5% saponin in DM) failed to reduce $CH_4$ emissions (g/kg DMI) in the experiment with non-lactating cows (n = 4 in experiment 3) and enhanced methanogenesis in the experiment with lactating cows (n = 8 in experiment 3). These results were in contradiction with previous data on sheep or cattle supplemented with tea saponin doses ranging between 0.05% up to ~0.40% of DM (Table 15). These differences may come from a bad quality of our tea saponin product and/or an alteration of the active compound during pelleting. Plant maturity, geographical area of production and extraction methods are three parameters affecting the final concentration and quality of the saponin (Li and Powers, 2012). In our experiments, we estimated the quantity of active compound in the tea saponin extract from the origin certificate of the Chinese supplier, but assessing the activity of our extract would have been useful before starting the trials. Moreover, we included the tea saponin extract in pelleted concentrates, as handling of the powder form led to respiratory irritation problems for users and feed refusals for animals. This issue has never been highlighted previously, whereas this plant extract was distributed as a powder and mixed with the diet in other studies (Table 15). We assume that pelleting denatured the active compound of tea saponin during heating (~40°C). A modification of the miscellaneous structure of *Quillaja* saponin was already observed after heating between 20 and 60°C (Mitra and Dungan, 1997).

In the present work, diet digestibility and N balance of non-lactating cows were unchanged by tea saponin supplementation (0.5% in DM). Inversely, ADF digestibility was improved by 8% when lactating cows were fed the same dose of this plant extract. To our

knowledge, this is the first report showing a positive effect of tea saponin on *in vivo* fiber digestibility. A positive effect of tea saponin on OM digestibility was reported *in vitro* (+21%; Wei et al., 2012), whereas no effect on nutrients digestibility was reported *in vivo* with goats supplemented with lower doses than those tested in our experiments (0.04 to 0.08% of DM; Zhou et al., 2012).

Tea saponin supplemented to lactating cows tended to reduce feed intake by 12% and significantly reduced milk yield by 18% compared to control, whereas this same plant extract did not affect DMI of non-lactating cows restricted fed. We assume that the lower intake explained the lower milk production as feed efficiency was similar between cows fed control with or without tea saponin. This finding agreed with Li and Powers (2012) who reported that tea saponin (0.11% in DM) reduced DMI of growing steers by 27% leading to a drop of their average daily weight gain of 80%. However, 0.4% tea saponin did not affect feed intake and growth of lambs (Mao et al., 2010).

**Overall results on the effects of tea saponin supplementation in diets of ruminants are contrasted. Additional research is necessary to give a reliable conclusion about its effect on animals' performances, diet digestibility and $CH_4$ emissions.**

### 2.1.3. Effect of nitrate fed individually to modify hydrogen consumption in the rumen on methane emissions and overall cows' performances

Nitrate (2.3% of DM) fed individually to non-lactating cows (n = 4 in experiment 1; n = 4 in experiment 3) never affected intake, total tract digestibility and N balance. Previous studies on sheep or cattle also reported the absence of nitrate effect on these parameters and animals' performances (Table 16), except for Hulshof et al. (2012; -6% DMI without affecting growth performance).

Nitrate decreased $CH_4$ emissions to a similar extent in our two experiments, with a reduction averaging 10.7% of $CH_4$ yield (g/kg DMI) per percent added nitrate. This result was in accordance with the literature: on average, $CH_4$ emissions were reduced by 10% per percent added nitrate, whatever the animal species and the nature of the basal diet (Table 16). Then, overall results show the efficiency and repeatability of the nitrate $CH_4$-mitigating effect between studies. Moreover, a recent meta-analysis reported a linear dose-response effect of nitrate (0.3 to 1.2 g/kg BW/day) on enteric $CH_4$ emissions with a reduction of 12% of $CH_4$ yield (g/kg DMI) per 0.1 g added nitrate/kg BW/day (Lee and Beauchemin, 2014b).

Kinetics of $CH_4$ emissions measured in open chambers indicated that dietary nitrate affected methanogenesis during the 3 h postfeeding in our experimental conditions (Figure 2, experiment 1). This result agreed with previous observations on sheep and cattle (Van Zijderveld et al., 2010; Van Zijderveld et al., 2011) and suggests that nitrate acts as a $H_2$-sink shutting down postprandial $CH_4$ production which is normally at its maximum.

**Overall results show that nitrate effect on $CH_4$ emissions is systematic and repeatable between studies, without altering digestive performances and N balance.**

### 2.1.4. Effect of association of strategies acting on hydrogen production and consumption in the rumen on methane emissions and overall cows' performances

We assumed that supplementing ruminants with $CH_4$-mitigating strategies acting on both production and use of $H_2$ reduces methanogenesis to a larger extent than when these strategies are fed individually. To test this hypothesis, two associations of strategies were tested on non-lactating cows: linseed plus nitrate (1.0% added fat plus 2.3% nitrate in DM; n = 4 in experiment 1) and tea saponin plus nitrate (0.5% saponin plus 2.3% nitrate in DM; n = 4 in experiment 3).

As tea saponin fed individually failed to decrease methanogenesis, we assumed that the observed $CH_4$ reduction (g/kg DMI; -28%) with tea saponin plus nitrate was fully explained by the nitrate effect. Consequently, this association of feeding strategies did not allow us to test our hypothesis and will not be further discussed.

For the first time, we observed a positive interaction between linseed and nitrate, as their association reduced $CH_4$ yield (g/kg DMI) by 32%. As these dietary strategies have different mechanisms of action, we expected a 39% $CH_4$ reduction for a fully additive effect (-17% and -22% $CH_4$ reduction for linseed and nitrate fed alone, respectively). According to stoichiometry and considering that control $CH_4$ emissions is equal to 100, $CH_4$ emissions corrected for the $CH_4$-mitigating effect of linseed fed individually (17%) would be $100 - 100 \times 0.17 = 83$. Then, these $CH_4$ emissions corrected for the $CH_4$-mitigating effect of nitrate fed individually (22%) would be $83 - 83 \times 0.22 = 65$. In total, this corresponds to an expected $CH_4$ reduction of 35% with linseed plus nitrate. But, the fat content in linseed plus nitrate was lower than in linseed fed individually (-1.6 % of DM), corresponding to a $CH_4$ mitigation potential of 10.7%. When applying the same stoichiometry estimation than previously, we obtained an expected $CH_4$ reduction of 27% with linseed plus nitrate. In both cases, observed $CH_4$ reduction with this dietary association was close to expected, and confirmed a fully

additive effect between these two strategies. This result is original and supports our initial assumption according to which decreasing $H_2$ pool in the rumen by acting on both $H_2$ production and consumption decreases $CH_4$ production to a higher extent than when acting on a single pathway. In addition, we showed that linseed plus nitrate (3.5% added fat plus 1.8% nitrate in DM) fed to lactating cows (n = 8 in experiment 2) during 4 months induced a constant reduction of $CH_4$ yield (g/kg DMI; -29%). This persistent effect showed the absence of adaptation of rumen microbiota. These results also suggest that the $CH_4$-mitigating effect of linseed plus nitrate is repeatable whatever the physiological stage of the cows.

Association of linseed (1.0% added fat in DM) to nitrate (2.3% of DM) did not modify N balance and total tract digestibility of non-lactating cows, confirming the effect observed when these dietary strategies were individually fed. Similarly, nitrate (1.8% of DM) plus linseed (3.5% added fat in DM) fed to lactating cows did not affect N balance, but tended to reduce ADF digestibility (-8%). This highlights the importance of studying the dose-response effect of this association on cattle digestibility.

Linseed plus nitrate supplemented to lactating cows tended to reduce *ad libitum* intake and milk production throughout our 4-month experiment. As feed efficiency (kg of milk per kg of feed) was similar between diets, we assumed that the lower intake explained the lower milk production. This is in contradiction with our results on non-lactating cows, for which we did not observe a detrimental effect of linseed plus nitrate on intake. As shown previously, in some cases, nitrate or linseed fed individually can reduce intake. Consequently, we showed that linseed plus nitrate is an efficient $CH_4$-mitigating strategy without improving cows' performances. We suggest that further studies should focus on the dose-response effect of this association on animals' performances.

**In conclusion, linseed plus nitrate is an efficient strategy to reduce $CH_4$ emissions in the long-term without altering digestive processes. However, the energetic benefits from the decreased $CH_4$ emissions did not appear beneficial for the dairy cows.**

### *2.2. Rumen fermentative and microbial mechanisms involved in selected methane-mitigating strategies*

To understand the mechanisms involved in the regulation of $H_2$ availability and $CH_4$ emissions, we studied the effects of the selected dietary strategies on rumen fermentation and microbiota.

2.2.1. Relationship between observed methane emissions and VFA profile

In the rumen, $H_2$ is mainly produced during acetate (C2) and butyrate (C4) synthesis, as two moles $H_2$ are generated per mole C2 or C4 produced. Inversely, the synthesis of one mole propionate (C3) or valerate (C5) consumes one mole $H_2$. Then a rise of C2 and/or C4 concentrations may indicate a higher $H_2$ availability in the rumen, whereas a rise of C3 and/or C5 concentrations may indicate a lower $H_2$ availability. As a result, knowing the key role of $H_2$ availability in methanogenesis, the ratio C2/C3 is positively correlated with $CH_4$ emissions expressed as a percentage of GEI (Sauvant et al., 2011; Figure 22).

Linseed fed alone to non-lactating cows (experiment 1) reduced C2/C3 ratio compared to control, *via* an increase of C3 concentration in the rumen. This result may explain a part of the observed $CH_4$-mitigating effect of linseed. To our knowledge, we are the first ones to report this effect, as most studies reported an absence of effect of linseed on rumen VFA composition (Chung et al., 2011; Doreau et al., 2009; Martin et al., 2011).

Tea saponin, fed alone to non-lactating or lactating cows (experiment 3) did not modify VFA profiles, except that it tended to increase C2/C3 ratio *via* a higher C2 concentration for lactating cows. This effect may explain why, for this particular group of cows, this plant extract led to higher $CH_4$ emissions compared to cows fed control treatment. Previous studies did not observe changes in VFA profiles in the rumen of sheep and goats supplemented with similar dosage of this plant extract (Mao et al., 2010; Yuan et al., 2007; Zhou et al., 2011).

Nitrate fed alone or in association with tea saponin (experiment 3) or linseed (experiments 1 and 2) to non-lactating or lactating cows increased C2/C3 ratio by increasing C2 or reducing C3 concentrations. These results confirmed previous findings reporting an increase of C2 and C4 concentrations and/or a decrease of C3 concentration in the rumen of animals fed this $H_2$-sink at a similar dosage (Hulshof et al., 2012; Nolan et al., 2010; Veneman et al., 2014). We assumed that this pattern is due to a reduction of $H_2$ availability in the rumen because of $H_2$ consumption for nitrate reduction. Acetate concentration may increase to compensate for the $H_2$ deficiency, and C3 concentration may decrease because of the lack of $H_2$ (Janssen, 2010).

For a more global approach, we related the observed C2/C3 ratios with $CH_4$ emissions (% of GEI) for each experiment and dietary treatment of this PhD thesis, and we compared these results with the relationship of Sauvant et al. (2011; Figure 22). Data from diets without $CH_4$-mitigating effect fit with the relationship, as low C2/C3 ratios were associated to low $CH_4$ emissions. Inversely, the highest C2/C3 ratios were observed with diets presenting the

best $CH_4$-mitigating effect (nitrate and/or linseed-supplemented diets). Then, the curvilinear positive relationship between $CH_4$ and C2/C3 ratio was not applicable in those cases.

**Results on control and tea saponin-supplemented diets confirmed the positive relationship between $CH_4$ emissions and VFA profiles. Nevertheless, this equation may be inaccurate with $CH_4$-mitigating dietary treatments such as linseed and nitrate-supplemented diets. This finding suggests that, in those specific cases, others interfering fermentative and/or microbial processes need to be taken into account to estimate $CH_4$ emissions.**

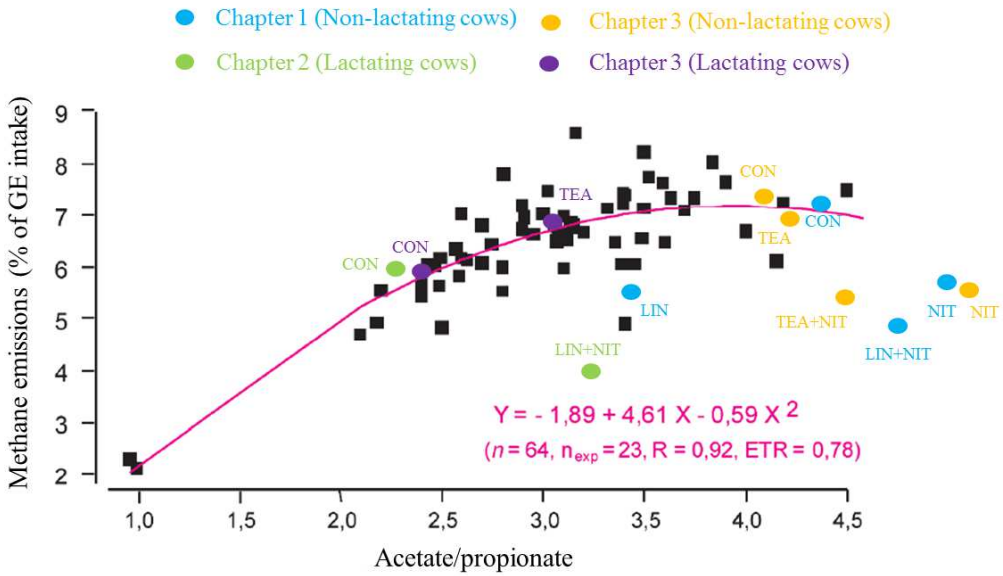


**Figure 22** Relationship between C2/C3 ratio and methane emissions (adapted from Sauvant et al., 2011). Colored points indicate the position of our data obtained in the four experiments of this PhD thesis (rumen samples taken 3 to 3.5 h following the morning meal).

### 2.2.2. Relationship between observed methane emissions and rumen microbiota

To our knowledge, the relationship between rumen microbial biomass synthesis and $CH_4$ emissions has never been studied. In this work, $CH_4$ emissions were reduced by linseed and nitrate fed alone or in association with linseed or tea saponin, whereas excretion of purine derivatives in the urine of non-lactating cows (experiments 1 and 3), as indicator of microbial biomass synthesis in the rumen, was not affected by dietary treatments (data not shown). We concluded that there was no relationship between rumen microbial biomass synthesis and $CH_4$ emissions in non-lactating cows (Figure 23).

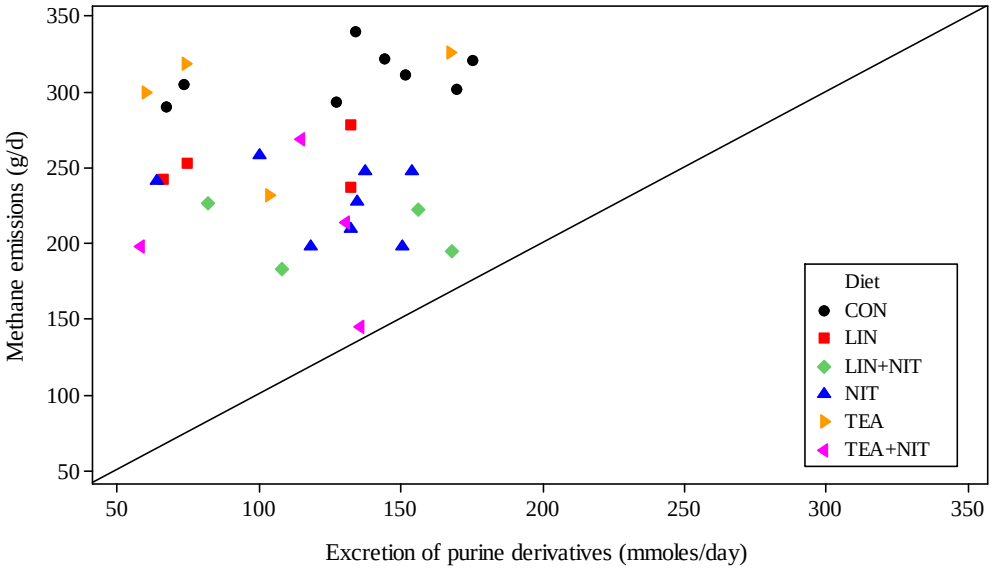


**Figure 23** Relationship between methane emissions and excretion of purine derivatives in the urine of non-lactating cows fed different dietary $CH_4$-mitigating strategies acting on the rumen hydrogen pool (experiments 1 and 3)

When looking at specific rumen microbial populations of these same animals, we observed that total bacteria concentrations were never affected by treatments (experiments 1 and 3). Compared to control diets, linseed fed alone to non-lactating cows (experiment 1) reduced protozoa (before feeding, -53%) and methanogens (after feeding, -8%) concentrations, while reducing $CH_4$ emissions (g/kg DMI) by 17%. The inhibiting effect of linseed towards protozoa was not observed when associating it with nitrate. For non-lactating cows, this may be caused by the lower dose of added fat in this diet (1.0% added fat in linseed plus nitrate *versus* 2.6% added fat in linseed). For lactating cows, this may be linked to a lower representativity of rumen samples taken by stomach tubing. In addition, rumen content was sampled after feeding whereas the defaunating effect of linseed fed alone was only observed before feeding. When relating observed $CH_4$ emissions with rumen protozoa concentrations obtained in cows fed linseed alone, we confirmed *in vivo* the positive relationship between these two parameters that we already highlighted in our meta-analysis (Guyader et al., 2014; Figure 24).

Tea saponin did not modify methanogens concentration or activity (experiment 3). Moreover, we did not observe the expected inhibiting effect on protozoa, explaining the absence of $CH_4$-mitigating effect of this plant extract fed to non-lactating and lactating cows after 4 weeks of feeding saponin. These results suggest an adaptation of rumen microbiota. Indeed, in sheep, a decrease of protozoa number after 4 days of feeding saponins (*Sesbania sesban*) was reported but this population recovered 10 days later (Newbold et al., 1997).

Nitrate fed alone or in association with linseed or tea saponin did not modify protozoa concentrations whereas $CH_4$ emissions (g/kg DMI) were reduced from -22 to -32%. Then, those dietary treatments confirmed that protozoa concentrations is not the only factor regulating methanogenesis and that other factors may be implied. Besides, quantity and activity of methanogens were reduced in the rumen of non-lactating cows fed diets including nitrate.

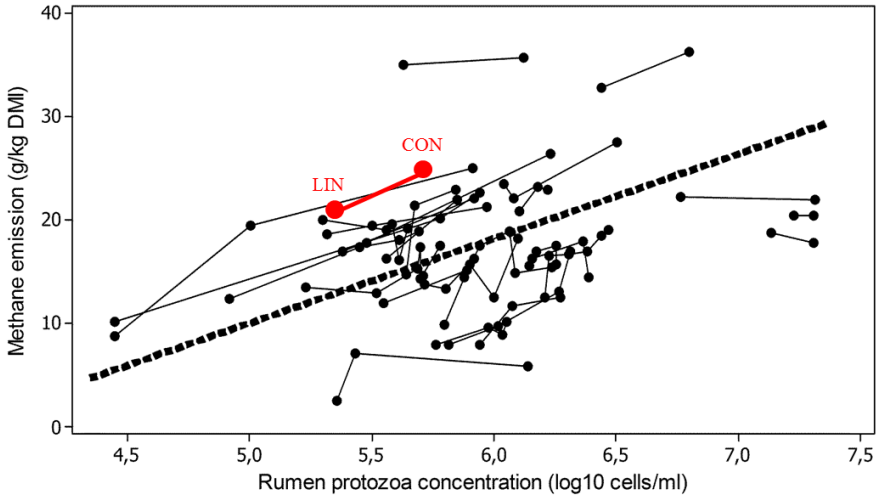


**Figure 24** Position of observed rumen protozoa concentration and methane emissions from non-lactating cows fed control diet (CON) or CON supplemented with 2.6% added fat (LIN) (experiment 1, this PhD thesis) among the experiments selected to study the relationship between these two parameters by meta-analysis (adapted from Guyader et al., 2014).

**These results confirm the importance of detailing rumen microbiota composition to understand the mechanisms involved in $CH_4$-mitigation. Such approaches should take into account the interactions between microbes and should describe the microbial populations in terms of quantity, activity and diversity.**

### 2.2.3. Nitrate reduction and lipids biohydrogenation: stoichiometric yield of methane reduction

In the rumen, it is commonly accepted that nitrate follows Dissimilatory Nitrate Reduction to Ammonium (DNRA), which consists in the reduction of 1 mole nitrate to 1 mole nitrite which is further reduced to 1 mole ammonia. The overall process consumes 4 moles $H_2$. Knowing that 4 moles $H_2$ are also required to produce 1 mole $CH_4$, it is considered that one mole added nitrate reduced $CH_4$ production by 1 mole, assuming a full conversion of nitrate to ammonia. In this PhD thesis, nitrate reduction (2.3% in DM) fed alone or in

association with tea saponin to non-lactating cows explained 82% of observed $CH_4$ reductions (g/day), which is close to the reported efficiencies in the literature (88% on average; Hulshof et al., 2012; Van Zijderveld et al., 2011; Veneman et al., 2013).

Rumen biohydrogenation of 1 mole C18:1, C18:2 or C18:3 consumes 1, 2 or 3 moles $H_2$, respectively. Then, assuming that a full biohydrogenation of unsaturated fatty acids occurs, one mole added C18:1, C18:2 or C18:3 reduced $CH_4$ production by 0.25, 0.50 or 0.75 moles. In the present work, biohydrogenation of lipids from linseed (2.6% added fat) fed alone to non-lactating cows only explained 11% of observed $CH_4$ reductions. This result is in accordance with a previous study on dairy cows fed a corn silage-based diet supplemented with 4.2 to 5.8% added fat from linseed (10% on average; Martin et al., 2008).

Fed to non-lactating cows, nitrate reduction (2.3% in DM) plus linseed biohydrogenation (1.0% added fat in DM) explained 72% of observed $CH_4$ reduction. Similarly, nitrate reduction (1.8% in DM) plus linseed biohydrogenation (3.5% added fat in DM) fed to lactating cows explained 46% of observed $CH_4$ reduction.

**We conclude that nitrate has a higher potential for $H_2$ consumption than PUFA. Nevertheless, this sole mechanism cannot fully explain the $CH_4$-mitigating effect of these dietary strategies.**

2.2.4. Relationship between methane emissions and gaseous hydrogen losses

In the literature, few studies simultaneously measured *in vivo* gaseous $H_2$ losses and $CH_4$ emissions on the same animals. Nevertheless, a negative relationship would exist *in vivo* between these two parameters. Indeed, sheep fed pelleted diets presented higher $H_2$ emissions than sheep fed fresh perennial ryegrass (0.115 *versus* 0.019% GEI), while emitting less $CH_4$ (Pinares-Patiño et al., 2010). Similarly, lactating cows supplemented with nitrate presented lower $CH_4$ emissions and higher $H_2$ emissions (0.017 *versus* 0.006% GEI) than when they were fed a control diet (Van Zijderveld et al., 2011). We assumed that gaseous $H_2$ emissions come from an excess of dissolved $H_2$ concentrations in the rumen. In addition to measuring $CH_4$ emissions, we monitored ruminal dissolved $H_2$ concentrations and gaseous $H_2$ emissions (data not show) of non-lactating cows fed a control diet with or without linseed (LIN, 2.6% added fat), nitrate (NIT, 2.3% nitrate) or linseed plus nitrate (LIN+NIT, 1.0% added fat plus 2.3% nitrate) (experiment 1). Animals fed diets including nitrate (NIT and LIN+NIT) presented higher dissolved $H_2$ concentrations (33.1 *versus* 3.8 µ*M* on average, respectively; Figure 5 in experiment 1) and gaseous $H_2$ emissions (4.5 L/h *versus* 0 L/h on average 1 h after

feeding, respectively) than animals fed CON and LIN. Then, similarly to gaseous $H_2$, we observed a significant negative relationship between $CH_4$ emissions (g/kg DMI) and dissolved $H_2$ concentrations (µ*M*) in the rumen of these animals (Figure 25): $CH_4 = 22.6^{***} - 0.181^{**} \times H_2$, with RMSE = 2.56 and $R^2 = 0.46$. This pattern may be explained by the toxic effect of nitrate on quantity and activity of methanogens, as reported for the first time in this work (experiments 1 and 3).

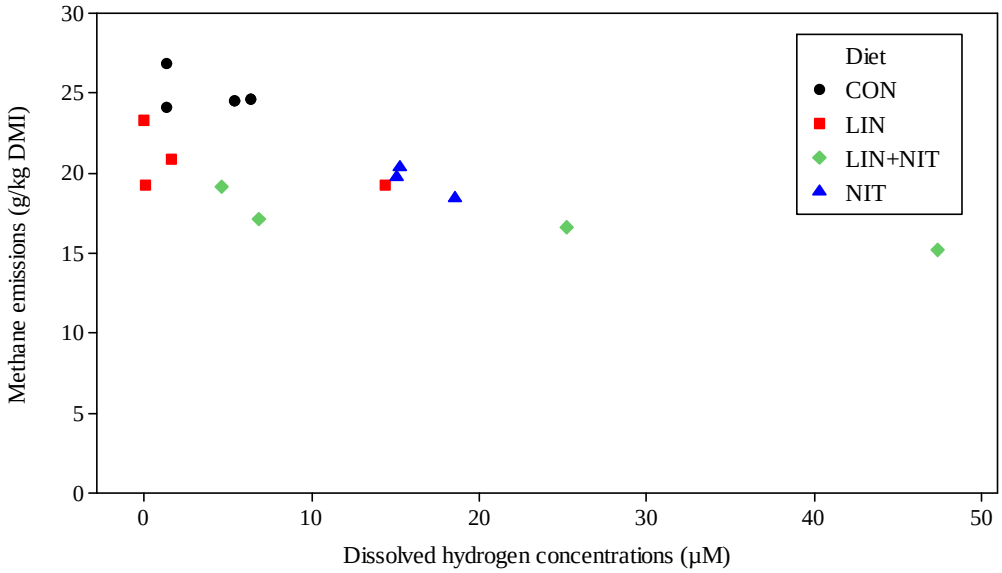


**Figure 25** Relationship between methane emissions and dissolved hydrogen concentrations in the rumen of non-lactating cows fed different dietary $CH_4$-mitigating strategies acting on the rumen hydrogen pool (experiment 1)

**Consequently, gaseous $H_2$ losses can occur when feeding animals with $CH_4$-mitigating strategies, but they represent small energetic losses and cannot by themselves explain observed $CH_4$ reductions.**

### *2.3. Overview of the mechanisms of action of dietary strategies: estimation of hydrogen distribution between rumen fermentation end-products*

To get a global view on the mechanisms of action of selected $CH_4$-mitigating dietary strategies (experiments 1, 2 and 3), we calculated the production and distribution of $H_2$ in the different rumen fermentation end-products (Figure 26). Production of $H_2$ was estimated from VFA and microbial biomass synthesis, knowing that 2 moles $H_2$ are generated per mole C2 or C4 produced, and 0.58 moles $H_2$ are produced per kg dry microbial matter growing on AA (Mills et al., 2001). Daily productions of individual VFA and dry microbial matter were

estimated from rumen fermentable organic matter content in diets and from microbial proteins production in the rumen (Nozière et al., 2010; Sauvant and Nozière, 2013). To estimate $H_2$ consumption, five pathways were considered: methanogenesis (4 moles $H_2$ / mole $CH_4$), VFA synthesis (1 mole $H_2$ / mole C3 or C5), microbial biomass synthesis (0.41 moles $H_2$ / kg dry microbial matter growing on NPN; Mills et al., 2001), nitrate reduction (1 mole $H_2$ / mole reduced nitrate) and lipids biohydrogenation (1, 2, and 3 moles $H_2$ / mole saturated C18:1, C18:2 and C18:3). We assumed that the totality of nitrate intake was reduced to ammonia *via* DNRA, and that the totality of C18:1, C18:2 and C18:3 intake was saturated during biohydrogenation. Detailed methods of calculations are given in Annex 2.

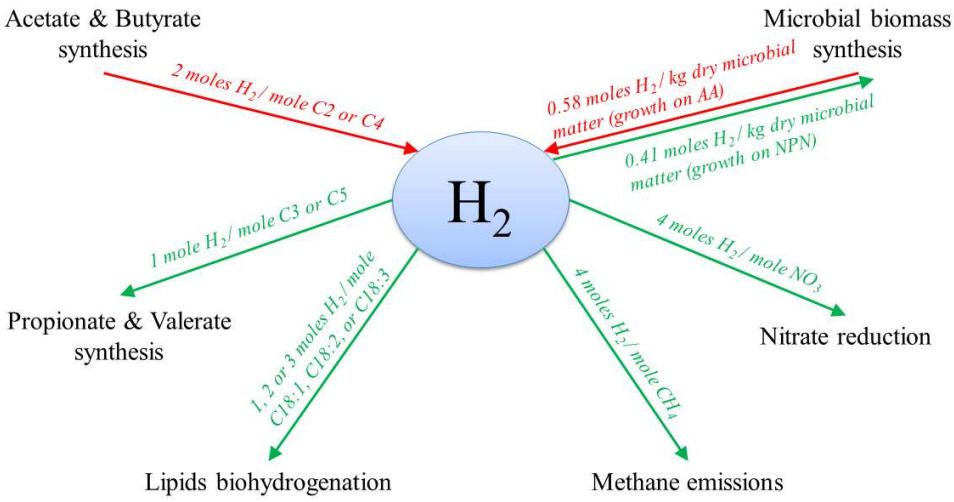


**Figure 26** Selected hydrogen producing (red arrows) and consuming (green arrows) pathways for estimation of hydrogen distribution between rumen fermentation end-products

To our knowledge, this work is the first to calculate production and distribution of $H_2$ in rumen fermentation end-products, when $CH_4$-mitigating strategies acting on ruminal $H_2$ availability are fed to non-lactating and lactating cows. The recovery rate of $H_2$ averaged 104 ± 11.2 %, which means that $H_2$ consuming pathways considered in our approach fully explained total $H_2$ produced. Among the different potential bias in each calculation step, one may come from the fact that $H_2$ production during dietary proteins fermentation was not considered, as this estimation would require more information on AA profile. Then, we can use these $H_2$ balances to summarize the mechanisms involved in the regulation of $H_2$ availability and $CH_4$ emissions by our tested $CH_4$-mitigating strategies (Figure 27; detailed data are provided in Annex 2)

In control diets, methanogenesis, C3 and C5 production, and microbial biomass synthesis respectively consumed 97.8% (91.1-102.1), 14.4% (10.5-18.7), and 0.33% (0.31-

0.36) of total produced $H_2$. Our *in vitro* approach (experiment 4) also gave similar results with control treatment (50% hay - 50% concentrate; 95 and 16% of produced $H_2$ were consumed for $CH_4$ and VFA production, respectively). These results were close to previous data obtained by a modelling approach, which reported that these fermentation pathways consume 48-80%, 19-33% and 0.6-12% of total consumed $H_2$ (Czerkawski, 1986; Mills et al., 2001). Overall results agree with the low contribution of microbial biomass synthesis in $H_2$ consumption and regulation of $H_2$ availability in the rumen.

As tea saponin supplementation poorly affected $CH_4$ emissions and rumen fermentation pathways, we logically did not find differences in the distribution of $H_2$ between fermentation end-products, compared to control treatments. Inversely, as reported in the analysis of VFA profiles, linseed fed alone (experiment 1) modified $H_2$ distribution between rumen fermentation end-products, as 13.3% of produced $H_2$ was directed towards C3 and C5 synthesis *versus* 10.5% in control treatment. A small part of produced $H_2$ was also used for lipids biohydrogenation (1.90%), confirming the low contribution of lipids biohydrogenation in direct $H_2$ consumption (1 to 2.6% reported in Czerkawski, 1986). To improve rumen $H_2$ balance with linseed, further approach should consider the inhibiting effect of PUFA on protozoa, which induced a lower $H_2$ production not taken into account in applied equations.

Nitrate reduction pathway consumed on average 21% of produced $H_2$ in the rumen. In diets including this $H_2$-sink, the sum of $H_2$ proportions directed towards nitrate reduction and methanogenesis was almost equal to the $H_2$ proportion directed towards methanogenesis in control diets. This highlights the equilibrium in the distribution of $H_2$ between these two pathways. To get a more precise rumen $H_2$ balance with nitrate, gaseous $H_2$ losses should be taken into account. Indeed, we observed that nitrate supplementation increased dissolved $H_2$ concentrations in the rumen (experiment 1), probably because of a direct toxic effect towards quantity and activity of methanogens (experiments 1 and 3). We assume that excess $H_2$ in the rumen was released in a gaseous form. Moreover, quantities of consumed $H_2$ during nitrate reduction in the rumen should be adjusted to take into account that a part of nitrate may have been converted to gaseous $N_2O$ produced *via* denitrification, as recently reported in cows (Neumeier et al., 2014; Petersen et al., 2014) and sheep (de Raphélis-Soissan et al., 2014). During denitrification, 2 moles nitrate are reduced to 1 mole nitrous oxide, while consuming 5 moles $H_2$.

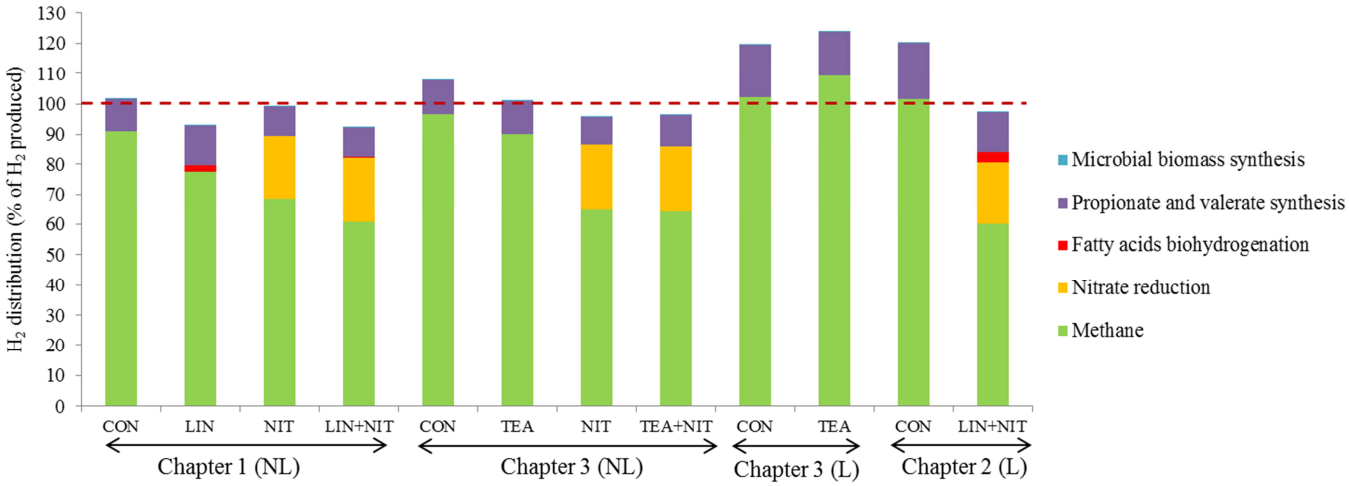


**Figure 27** Estimation of hydrogen distribution (% of produced hydrogen) between rumen fermentation end-products in the experiments of this PhD thesis. Detailed figures are given in Annex 2.

**In conclusion, mechanisms of selected $CH_4$-mitigating strategies involved modifications in rumen fermentation processes related to shifts in microbiota. This work confirms the interest of simultaneous study of fermentative and microbial parameters, in order to understand the mechanisms involved in the regulation of rumen $H_2$ availability.**

## III. PRACTICAL USE OF ASSOCIATION OF METHANE MITIGATING STRATEGIES ACTING ON HYDROGEN PRODUCTION AND CONSUMPTION: FOCUS ON LINSEED PLUS NITRATE

While testing the $CH_4$-mitigating effect of linseed plus nitrate, we showed that acting on both $H_2$ production and consumption decreased methanogenesis to a higher extent than when acting on a single pathway. However, before considering the practical use of this dietary strategy at the farm scale, several recommendations deserve to be highlighted.

### *3.1. Animals' health and zootechnical performances*

#### 3.1.1. Is nitrate a safe non-protein nitrogen source in substitution for urea ?

Knowing the low efficiency of N utilization in ruminants (25% on average; Calsamiglia et al., 2010), one may ask about the effect of feeding nitrate on animals' N metabolism. Indeed, potential risk of using nitrate as a NPN source substituting urea is a low

utilization by the animal resulting in additional N release in the form of nitrate, nitrite or ammonia, which would contribute to N pollution from agriculture. However, we confirmed that nitrate supplemented to non-lactating or lactating cows did not increase the quantity of N excreted in urine, feces and milk compared to cows supplemented with urea (Van Zijderveld et al., 2011). Then, to avoid excessive N losses, we recommend nitrate supplementation in substitution for urea to animals fed diets not already containing nitrate (such as nitrate-fertilized pasture) or diets deficient in degradable N (such as corn silage, sugar cane, sugar beet, molasses or cassava-based diets) (Leng, 2008).

Potential risk of nitrate poisoning of animals is one of the major limitations of its utilization in animal nutrition. Indeed, in the rumen, nitrate is converted to nitrite and then ammonia. If nitrite accumulates in the rumen, it can pass through the rumen wall into the blood and convert Hb to metHb, which cannot then transport oxygen to the tissues (Lewis, 1951). The level of blood metHb determines the symptoms severity: first symptoms are depressed feed intake, milk production and weight gain, then animals become more susceptible to infections, have more reproductive failure and present brown mucous membrane discoloration, to finish with respiratory distress, coma, cyanosis, and even death (Bruning-Fann and Kaneene, 1993).

In this work, nitrate was gradually introduced in the diet of cows (up to 2.3% in DM) during a 10 to 15-day adaptation period. During this period, we observed a gradual increase of blood metHb levels, without apparition of clinical symptoms. Following this period, blood metHb recovered low levels situated between 1.2 and 10.5% on average. We also showed the absence of nitrate poisoning during its long-term (4 months) supplementation to lactating cows. Our data were in accordance with the literature on cattle, but higher than data reported on sheep fed doses close to our experimental conditions (Table 17). These high values of metHb in our experiments are difficult to explain, but may come from a combination of several factors such as animal species, length of adaptation period, and feeding frequency. Cattle would be more susceptible to nitrate poisoning compared to sheep (Leng, 2008). In addition, within a species, some animals would have more risks of developing methemoglobinemia: erythrocytes (red blood cells) phenotype would affect activity of the enzyme responsible for metHb reduction (Godwin, 2014). Our adaptation period was shorter than in other experiments from the literature, and animals acclimatized to nitrate during a long adaptation period have lower risks of blood metHb (Lee and Beauchemin, 2014b). In the present work, a restricted feeding was always applied. However, for a same amount of nitrate in diet, a fractionated feeding throughout the day limits the risk of blood metHb *via* a slow

release of the additive in the rumen (Figure 28; Callaghan et al., 2014). With the same mechanism, *ad libitum* feeding reduces the risk of blood metHb compared to restricted feeding.

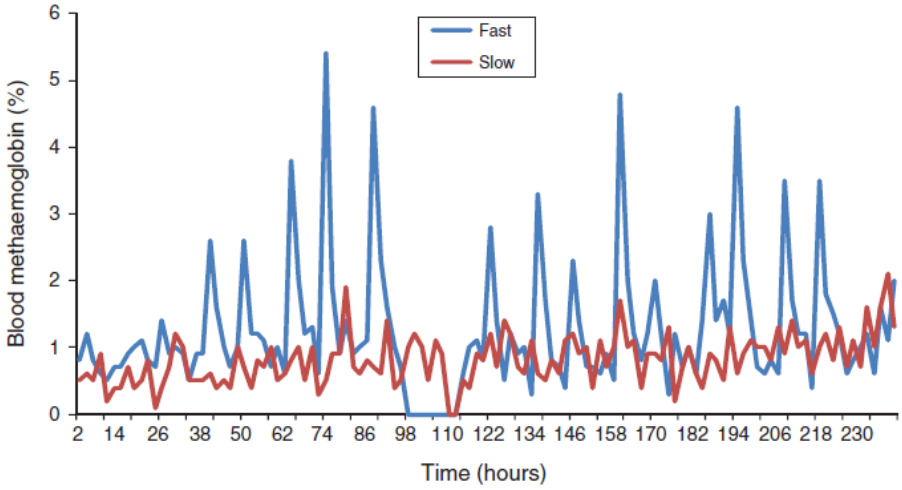


**Figure 28** Effect of feeding a same amount of nitrate to steers consuming the dose within 5 min (fast) or 45 min (slow) on blood methemoglobin (from Callaghan et al., 2014)

Then, from a practical point of view, we emphasize the importance that farmers do not directly deal with nitrate utilization, to avoid its excessive and uncontrolled distribution. Solutions must be thought according to feeding frequency, in order to match rumen $H_2$ production from feed fermentation with nitrate concentration. For animals continuously eating small quantities of feed, solutions would consist in the use of nitrate-supplemented lick blocks. However, because of the uncontrolled and variable access between animals, the use of slow-release encapsulated nitrate may be a safer solution, and gave similar extent of $CH_4$ abatement without raising blood metHb levels (El-Zaiat et al., 2014). For animals eating their meals in a fractionated manner, one may consider including the nitrate in the TMR in its raw form or included in pellets as performed in this PhD thesis.

**Table 17** Compiled data from the literature on the effects of nitrate supplementation to cattle or sheep on blood methemoglobin levels

| Reference | Animal species | Forages (% of DM) | Voluntary intake (%) | Time before full dose (days) | Nitrate dose (% of DM) | Blood sampling (h after feeding) | Average metHb[1] (%) | Maximum metHb[2] (%) |
|---|---|---|---|---|---|---|---|---|
| Experiment 1, this PhD thesis | Dry cows | Grass hay (50) | 90 | 10 | 2.3 | 3 | 10.5 | 26.3 |
| Experiment 2, this PhD thesis | Dairy cows | Corn silage, grass hay (60) | 95[3] | 14 | 1.8 | 3.5 | 1.2 | 30.8 |
| Experiment 3, this PhD thesis | Dry cows | Grass hay (50) | 90 | 10 | 2.3 | 3 | 4.5 | 25.9 |
| Sar et al., 2004 | Adult sheep | Timothy/lucerne hay (80) | Maintenance level | 7 | 0.7 | NA | 9.2 | 18.4 |
| Nolan et al., 2010 | Adult sheep | Oaten hay (100) | NA | 18 | 2.5 | NA | 0.6 | 2.8 |
| Van Zijderveld et al., 2010 | Adult sheep | Corn silage, barley straw (90) | 95[3] | 21 | 2.6 | 3 | 0.5 | 7.0 |
| Van Zijderveld et al., 2011 | Dairy cows | Corn silage, dried alfalfa, barley straw (66) | 95 | 21 | 2.1 | 3 | 3.9 | 19.0 |
| Li et al., 2012 | Lambs | NA | Ad libitum | 7 | 2.3 | 3 | 0.6 | 1.2 |
| de Raphélis-Soissan et al., 2014 | Adult sheep | Oaten hay (100) | Maintenance level | 14 | 2.0 | 2.5 | 14.0 | 45.0 |
| El-Zaiat et al., 2014 | Lambs | Grass hay (60) | Ad libitum | 21 | 3.4 | 6 | 1.08 | <1.1 |
| Lee et al., 2014b | Steers | Forage (55) | 75 | 20 | 0.7<br>1.3-1.9<br>2.6-3.9 | 3 | <1<br>8.6<br>3.3 | <1<br>23.6<br>13.6 |
| | Steers | Forage (55) | Ad libitum | 21 | 2.0 | NA | 8.4 | 23.6 |

NA: Data not available

[1] After adaptation period

[2] Throughout the experiment

[3] *Ad libitum* during adaptation period, 95% restricted during measurement weeks

#### 3.1.2. Required research on nutrients digestibility and zootechnical performances

In this PhD thesis, linseed plus nitrate fed to non-lactating (1.0% added fat plus 2.3% nitrate in DM) or lactating (3.5% added fat plus 1.8% nitrate in DM) cows did not modify total tract digestibility of DM, OM and NDF compared to control diets. Nevertheless, this association tended to reduce ADF digestibility, intake and milk production of dairy cows, even if feed efficiency was similar between diets. Then, before using association of linseed plus nitrate as a $CH_4$-mitigating feeding strategy at the breeding scale, an additional dose response study is required to determine the optimal dosage for maintaining animals' performances.

To our knowledge, the impact of nitrate supplementation on reproduction performances of cows still requires further research. Indeed nitrate has been reported to lower conception rate (0.7 mg/kg BW; Davison et al., 1964) and to cause abortions in beef and dairy cattle (Sonderman and Odde, 1993). The death of the fetus would be induced by a decrease in oxygen concentration in fetal arterial blood in dams fed nitrate and by a rise of nitrate concentration in the placenta.

### *3.2. Quality of animals' products and societal perception*

#### 3.2.1. Benefits of linseed and nitrate for quality of animals' products

In addition to be an efficient $CH_4$-mitigating strategy, linseed plus nitrate may improve the quality of milk and meat from ruminants. Indeed, previous studies reported that linseed supplementation improves milk and meat fatty acids profiles by increasing the quantities of PUFA, which have well-known positive effects on human health (anticarcinogenic and antiatherogenic; Chilliard et al., 2009; Scollan et al., 2001). Besides, the advantages of using linseed in animal feed are largely promoted by private companies, such as in the French initiative "Bleu-Blanc-Coeur". To complete the present work, characterization of milk fatty acids profile of samples taken from dairy cows fed linseed plus nitrate is under progress.

Concerning nitrate, a potential risk of its supplementation would be the accumulation of nitrate and nitrite in animals' products for human consumption. Indeed, even if nitrite is a common food preservative, an excess of nitrite in humans diet may promote gastric inflammation (Weitzberg and Lundberg, 2013). For the first time, we reported the absence of nitrate and nitrite residues in milk and home-made milk products (yoghurts, whey, curd and 6-wk ripened Saint-Nectaire cheese) from cows fed nitrate (1.8% of DM) plus linseed (3.5% added fat in DM; experiment 2) during 4 months. This result completes the work carried out

by El-Zaiat et al. (2014), who also did not detect nitrate and nitrite residues in meat of lambs fed nitrate (3.4% of DM). Consequently, based on current knowledge, the consumption of milk and meat from animals fed linseed plus nitrate does not seem to be an issue for human health.

#### 3.2.2. Negative perception of nitrate by consumers and farmers

In public opinion, nitrate is viewed as a chemical product used as a crop fertilizer, and is frequently associated with water pollution and health hazards. Then, despite the absence of risks in the consumption of milk and meat from cows fed linseed plus nitrate, one may expect some hesitation of consumers to buy such products. Trainings and dialogue with them may reduce their time for acceptance of this dietary strategy. From farmers point of view, knowing the severe legislation on agricultural nitrate release (EU nitrate directive 91/676/EEC), they may apprehend using this additive in animals' diets, even if it does not induce additional N losses. Moreover, as the relationship between $CH_4$ emissions abatement and improvement of animals' performances has never been reported, farmers' willingness to participate in the global effort of $CH_4$ mitigation may be only enhanced if they receive direct governmental subsidies. However, in the case that emissions taxes would be implemented, the major difficulty for governments would be the on-farm measurement of $CH_4$-emissions (Gerber et al., 2010). Anyway, to our knowledge, in the French and European legislations, nitrate has been authorized as a raw feed material, but not as an animal feed additive, even if several reports support its utilization as a $CH_4$-mitigating strategy at national (Doreau and Benoît, 2013) or international (Gerber et al., 2013a) levels.

### *3.3. Environmental benefits of using linseed plus nitrate: importance of a global approach*

We reported the long-term (4 months) $CH_4$-mitigating effect of linseed plus nitrate, which suggests that rumen microbiota do not adapt to this dietary treatment and supports its application at the farm scale. However, to consider applicability of this dietary strategy, two other environmental criteria remain to be discussed.

#### 3.3.1. Nitrous oxide emissions

Nitrate supplementation may induce $N_2O$ emissions from the ruminants and/or from manure fermentation, if excessive dietary nitrate is released in urine (de Raphélis-Soissan et al., 2014; Neumeier et al., 2014; Petersen et al., 2014). Nitrous oxide is the third GHG at the

global level (8% of total GHG produced), with a GWP of 298 (IPCC, 2007). Then, we highly encourage further studies to monitor $N_2O$ emissions to assess the global GHG mitigating efficiency of linseed plus nitrate. To our knowledge, only one study used this type of approach, and showed that the $CH_4$-mitigating efficiency of nitrate was lowered by 18% due to the rise in $N_2O$ emissions from eructation or manure of sheep supplemented with this additive (de Raphélis-Soissan et al., 2014).

### 3.3.2. Environmental effectiveness of linseed and nitrate production

To assess applicability of wide scale supplementation of linseed plus nitrate in ruminants' nutrition, it will be important to analyze its global effect on GHG emissions at the chain level (from feed production to the farm gate) *via* a life cycle assessment (LCA). By this approach, one study already reported the effect of individual supplementation of extruded linseed (1.1% added fat in DM for summer; 2.8% added fat in DM in winter) and nitrate (1.0% in DM in summer and winter) on GHG changes at the farm scale, using a Dutch dairy farm model (Van Middelaar et al., 2014). They assume that 1% added fat or nitrate reduced enteric $CH_4$ emissions by 6.1 and 9.4% on average, respectively. Compared to a reference dairy farm (840 kg $CO_2$-equivalents/T fat and protein-corrected milk), supplementation of extruded linseed reduced emissions by 9 kg $CO_2$-equivalents/T fat and protein-corrected milk, whereas supplementation of nitrate reduced emissions by 32 kg $CO_2$-equivalents/T fat and protein-corrected milk.

With a more global approach, Doreau et al. (2014) assessed the national potential abatement of $CO_2$-equivalents up to year 2030 if French cattle was supplemented with either additional fat (whatever the source; 3.5% added fat in DM only for cows receiving more than 1 kg concentrate daily) or nitrate (1% in DM only for cows receiving diets short in fermentable protein). They assumed a mean abatement of enteric $CH_4$ emissions of 4 and 10% per percent added fat and nitrate, respectively. They resulted that, at the French scale, fat may present a higher GHG abatement potential than nitrate (1.89 M T $CO_2$-equivalents *versus* 0.48 M T $CO_2$-equivalents in 2030). Similar approaches need to be considered to assess the global environmental impact of linseed plus nitrate supplementation to cattle, but we assumed an additive positive effect between these two dietary strategies.

### *3.4. Economical aspect*

The final aspect to assess applicability of a $CH_4$-mitigating strategy is the cost effectiveness (€/T $CO_2$-equivalents reduced) of its application, which is calculated by dividing the decrease in labor income of farm (€/year) by the decrease in GHG emissions at the chain level (kg $CO_2$-equivalents/year) (Van Middelaar et al., 2014). At the farm scale with a Dutch dairy farm model, nitrate (1.0% in DM in summer and winter) supplementation would be more cost-effective than extruded linseed (1.1% added fat in DM for summer; 2.8% added fat in DM in winter) supplementation (241€/T $CO_2$-equivalents reduced *versus* 2,594€/T $CO_2$-equivalents reduced; Van Middelaar et al., 2014).

At the national scale, Doreau et al. (2014) confirmed these results using the French model. They first showed that fat supplementation to ruminants is the best strategy for global abatement of GHG emissions in French agriculture, even if it is the most expensive one (Figure 29). This would be mainly due to high production costs and poor availability of raw material causing high importation costs. They also reported that nitrate (1% in DM only for cows receiving diets short in fermentable protein) supplementation is more cost-effective than fat (3.5% added fat in DM only for cows receiving more than 1 kg concentrate daily) supplementation (38€/T $CO_2$-equivalents reduced *versus* 267€/T $CO_2$-equivalents reduced). Both studies cited above highlighted a range of uncertainties in their calculations, because of variability in feed prices which has a strong impact on costs of option (Doreau et al., 2014a). From these results, we suggest that linseed plus nitrate supplementation to cattle would be an expensive $CH_4$-mitigating option, even if it would result in a high annual abatement of $CO_2$-equivalents.

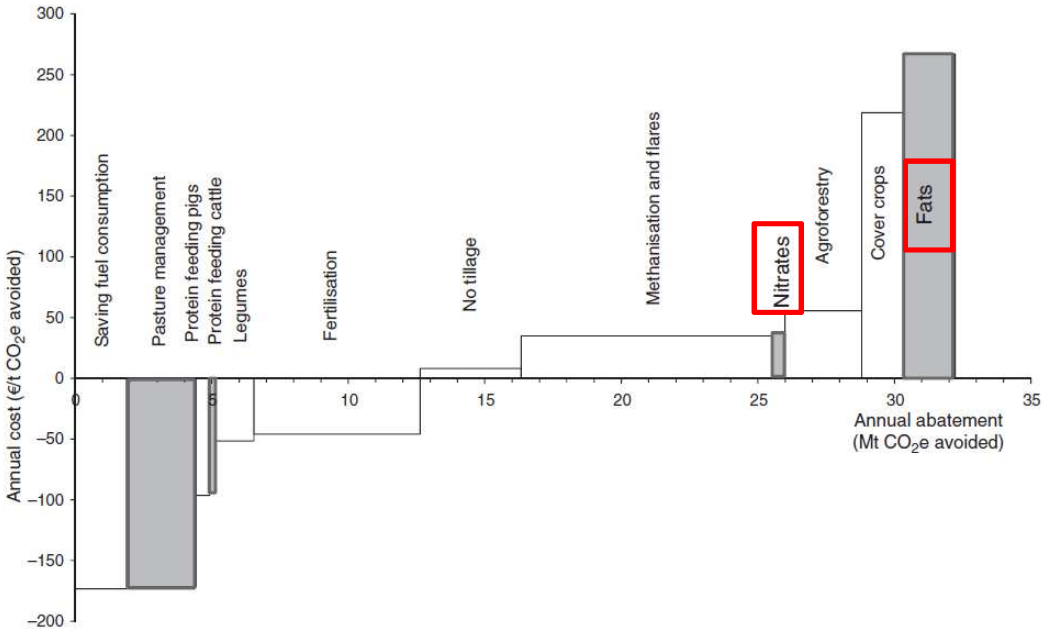


**Figure 29** Place of several options for enteric methane mitigation in a global abatement cost curve for French agriculture (from Doreau et al., 2014a)

**We conclude that linseed plus nitrate can be proposed as a $CH_4$-mitigating strategy in ruminant nutrition under controlled conditions. Linseed already has a good public image thanks to its positive effect on quality of ruminants' end-products. For the first time, we showed that the consumption of dairy products from nitrate-fed animals does not seem an issue for the human health. Further work should detail the cost-effectiveness of this strategy.**

# Conclusions and perspectives

This PhD thesis deepened the knowledge about the importance of the different metabolic pathways of $H_2$ in the rumen, in order to propose and evaluate new dietary strategies to mitigate $CH_4$ emissions in ruminants. We assumed that manipulating at the same time production AND utilization of $H_2$ in the rumen allows a more important reduction of $CH_4$ emissions than acting on a single pathway (production OR utilization). With the bibliographical approach, we selected dietary strategies with different modes of action on rumen $H_2$ metabolism: lipids from linseed or tea saponin for their potential to decrease $H_2$ production through their toxic effect on protozoa, and chemical components such as nitrate for their potential to consume $H_2$ without affecting protozoa. To test our hypothesis, these strategies were fed alone or in association to non-lactating and lactating cows. Tea saponin plus nitrate did not allow us to accept or refuse our hypothesis, as tea saponin had no effect on rumen protozoa concentrations. On the contrary, we reported a fully additive and long term $CH_4$-mitigating effect of linseed plus nitrate. To complete this work, several perspectives can be drawn to improve knowledge on involved mechanisms, and to study the on-farm applicability of using association of dietary treatments acting differently on the rumen $H_2$ pool.

## I. DEEPER CHARACTERIZATION OF THE RUMEN MICROBIOTA INVOLVED IN HYDROGEN METABOLISM AND METHANE PRODUCTION

Few studies have related in the same trial variations of $CH_4$ production with the characteristics of the ruminal microbial ecosystem. The originality of our approach will be to combine a quantitative approach (daily production of $CH_4$) to a cognitive approach (microbial parameters) of digestive processes in order to understand the observed phenomena. We already reported that linseed reduced protozoa ($H_2$-producers) and methanogens ($H_2$-consumers) concentrations, and that nitrate inhibited quantity and activity of methanogens without influencing genes coding for microbial nitrate or nitrite reductases. To deepen this work, we aimed at assessing the effect of the different tested $CH_4$-mitigating strategies tested on non-lactating cows (experiments 1 and 3) on rumen meta-transcriptome (functional diversity, ARN) using the MiSeq technology of Illumina and by targeting together bacteria, archaea and protozoa as applied previously on DNA (Kittelmann et al., 2013; Annex 1). This approach was unfortunately unsuccessful, for unknown reasons. Work is now under progress to analyze, by the same approach, the rumen meta-genome (sequences diversity, DNA) of

bacteria, archaea and protozoa in these same samples. The integration of overall collected data will allow a better understanding of ruminal methanogenesis and associated biological phenomena.

## II. STUDY OF THE EFFECT OF PELLETING PROCESS ON TEA SAPONIN

Tea saponin included in a pellet failed to reduce methanogenesis of non-lactating and lactating cows. We explained this result by the absence of effect of this plant extract on protozoa. We suspect that the plant active compound was denatured during granulation. To check this hypothesis, an *in vitro* experiment will be carried out soon. The effect of two tea saponin forms (powder *versus* pelleted) at different doses will be tested on $CH_4$ production and protozoa concentrations after 24 h *in vitro* incubation with rumen inoculum from cattle. If it turns out that it is the pelleting process which denatured the substance, one can consider further research to develop solutions for a better ingestion of tea saponin by animals without prior process.

## III. IMPROVEMENT OF LINSEED PLUS NITRATE ACCEPTABILITY

Linseed plus nitrate persistently decreased methanogenesis. However, the energetic benefits from the decreased $CH_4$ emissions did not appear beneficial for the animal. On the contrary, linseed plus nitrate tended to reduce animals' digestibility and performances. Solving this issue is essential for on-farm acceptance of this dietary strategy. More studies are also required to secure the mode of distribution of nitrate, which may lead to animals' health issues when quickly ingested. Additional research on genetic selection of animals presenting lower risks of developing metHb may also be considered. At the consumer level, acceptance of linseed plus nitrate in ruminants' nutrition will be facilitated if the beneficial effect of linseed on the nutritional value of animals' products and if the absence of nitrate residues in animal products is confirmed. In this objective, systematic control of the quality of animals' products has to be considered.

## IV. OPENING TO OTHER ASSOCIATION OF DIETARY STRATEGIES ACTING ON THE RUMEN HYDROGEN POOL

This PhD thesis showed that the association of dietary strategies having different mechanisms of action to reduce $H_2$ availability in the rumen reduced $CH_4$ emissions to a greater extent than when strategies were fed individually. Then, this work opens up the field of possibilities about testing other association of strategies. Linseed may be replaced by other lipids sources such as grape marc, sunflower or canola seeds, which $CH_4$-mitigating effect has already been reported. Nitrate may be replaced by other additives known to modify $H_2$ consumption such as sulfate, nitro-ethane or nitro-oxypropanol. Electrons acceptors such as iron or manganese still require further research. In any cases, for on-farm applicability, a $CH_4$-mitigating dietary strategy has to be efficient on the long term with no adverse effect on animals' health, performances and products quality for human consumption. In addition, life cycle assessment should be applied to analyze the cost and environmental effectiveness of the selected dietary strategy.

# List of publications

*Peer-reviewed international scientific journals:*

**Guyader J**, Eugène M, Nozière P, Morgavi DP, Doreau M, Martin C 2014. Influence of rumen protozoa on methane emissions in ruminants: A meta-analysis approach. Animal 8: 1816-1825.

**Guyader J**, Eugène M, Meunier B, Doreau M, Morgavi DP, Silberberg M, Rochette Y, Gerard C, Loncke C, Martin C. Additive effect between dietary linseed oil and nitrate as methane emission-reducer in cattle. Journal of Animal Science (Accepted, in revision).

**Guyader J**, Doreau M, Morgavi DP, Gerard C, Loncke C, Martin C. Long-term methane mitigating effect of linseed plus nitrate supplemented to dairy cows. Journal of Dairy Science (Accepted, in revision).

**Guyader J**, Eugène M, Doreau M, Morgavi DP, Gerard C, Loncke C, Martin C. Absence of methane mitigating effect of tea saponin fed to non-lactating and lactating cows. (In progress).

**Guyader J**, Silberberg M, Popova M, Morgavi DP, Seradj AR, Gerard C, Loncke C, Martin C. Dietary nitrate inhibits rumen methanogenic archaea without influencing genes coding for microbial nitrate or nitrite reductases. (In progress).

**Guyader J**, Tavendale M, Martin C, Muetzel S. Dose response effect of nitrate on hydrogen distribution between rumen fermentation end-products: an in vitro approach. Animal Feed Science and Technology (In preparation).

*Scientific communications in international congress:*

**Guyader J**, Eugene M, Doreau M, Rochette Y, Morgavi DP, Martin C 2014. Association of nitrate and linseed oil effectively reduces methane emission in ruminants. Proc. Aust. Soc. Anim. Prod. 30, 87. ISNH-ISRP, 8-12 September 2014, Canberra, Australia (poster)

**Guyader J**, Silberberg M, Morgavi DP, Martin C 2014. Postprandial kinetics of dissolved hydrogen in the rumen of cows fed nitrate and/or linseed oil. Proc. Aust. Soc. Anim. Prod. 30, 86. ISNH-ISRP, 8-12 September 2014, Canberra, Australia (poster)

**Guyader J**, Eugène M, Nozière P, Morgavi DP, Doreau M, Martin C. Influence of rumen protozoa on methane emissions in ruminants: a meta-analysis approach. Greenhouse Gases and Animal Agriculture, 24-26 June 2013, Dublin, Ireland (oral communication)

*Scientific communications in national congress:*

**Guyader J**. Manipulation du pool d'hydrogène disponible dans le rumen pour réduire la production de méthane chez le bovin. Conseil Scientifique Unité Mixte de Recherche sur les Herbivores de l'INRA Theix, 1st June 2012 (oral communication)

**Guyader J**, Eugène M, Nozière P, Morgavi DP, Doreau M, Martin C. Influence des protozoaires du rumen sur les émissions de méthane par les ruminants: Approche par méta-analyse. Conseil Scientifique Unité Mixte de Recherche sur les Herbivores de l'INRA Theix, 5 february 2013 (oral communication)

**Guyader J**, Eugène M, Nozière P, Morgavi DP, Doreau M, Martin C. Influence des protozoaires du rumen sur les émissions de méthane par les ruminants: Approche par méta-analyse. Journées de l'Ecole Doctorale SVSAE, Université Blaise Pascal, Clermont-Ferrand, 13 june 2014 (oral communication)

# Annex

# I. ANNEX 1 - ANALYSIS OF RUMEN MICROBIOTA DIVERSITY BY HIGH THROUGHPUT SEQUENCING METHODS

Total nucleic acids (DNA and RNA) were co-extracted from rumen samples taken from non-lactating cows (experiments 1 and 3) preserved with RNA*later*® and stored at -80°C (Popova et al., 2011). RNA reverse-transcribed to cDNA was used to describe the rumen functional diversity by targeting the cDNA copies of 16S (bacteria, archaea) or 18S (protozoa) rRNA.

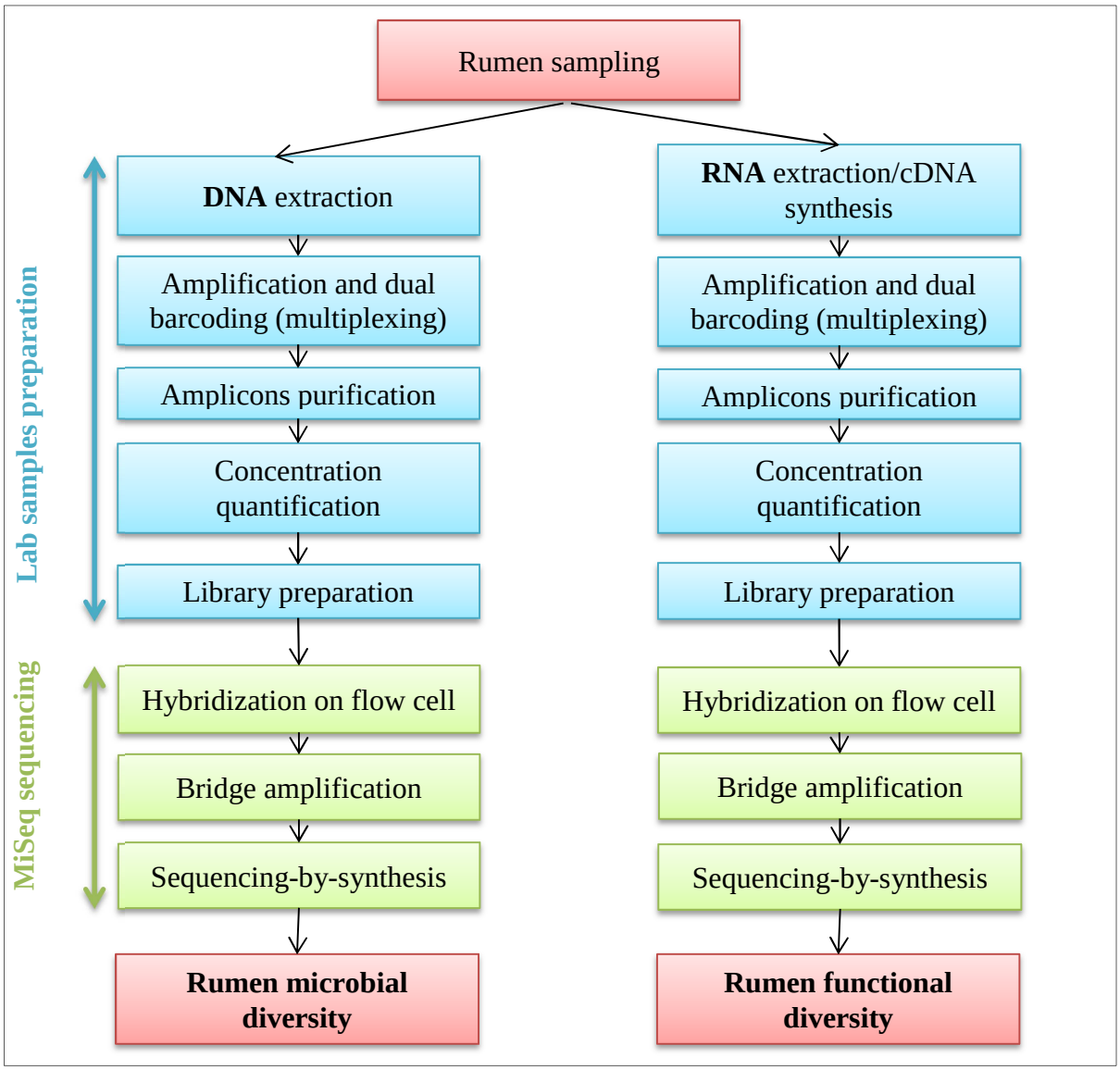


**Figure 1** Framework of samples preparation and analysis of rumen microbiota diversity with MiSeq technology (Illumina)

### *1.1. Samples preparation (Figure 1)*

Separate PCR were run in duplicate for each target species (bacteria, archaea and protozoa) and using for each sample: 5 µL PCR Buffer (10X), 6 µL $MgCl_2$ (25 m*M*), 1 µL dNTPs, 2.5 µL forward and 2.5 µL reverse primers (10 p*M*), 0.25 µL HotStar Taq DNA polymerase Taq, 1 µL cDNA template and 31.75 µL water molecular biology grade. Each forward and reverse primers contained (Figure 2): i) an Illumina adaptor (5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-3' and 5'- CAA GCA GAA GAC GGC ATA CGA GAT-3', respectively) common to the three target species, ii) a unique 8-base barcode for multiplexed sample identification (Kozich et al., 2013), iii) a 10-base pad common to the three target species for limiting primer dimmers (5'-TAT GGT AATT-3' and 5'-AGT CAG TCAG-3', respectively), and iv) the group-specific primer (Table 1) with a 2-base linker specific for each target species. The pad sequence was selected so that the combined pad, linker, and gene-specific primer would have a melting temperature over 60°C. Amplification program consisted of one denaturation step (95°C, 15 min), 30 cycles of denaturation (95°C, 20 sec), touchdown annealing (65°C to 55°C, 30 sec) and elongation (72°C, 5 min), and one final elongation step (72°C, 10 min). Theoretical lengths of amplicons were ~364, 309 and 355 base pairs (bp) for bacteria, archaea and protozoa, respectively. The duplicate PCR products were pooled to obtain a final volume of 100 µL.


**Figure 2** Dual barcoded primers used for multiplexed sequencing with MiSeq technology. Forward and reverse barcodes combination is different for each sample and target species. Linker and primers are similar among samples but different between target species.

**Table 1** Primers used for analysis of diversity of rumen microbiota by MiSeq technology

| Organism-Target region (Reference) | Primer set | (Linker)-Primer sequences 5'-3' |
|---|---|---|
| Bacteria-16S | S-DBact-0564-a-S-15 | (GT)-AYTGGGYDTAAAGNG |
| (Klindworth et al., 2013) | S-DBact-0785-b-A-18 | (CC)-TACNVGGGTATCTAATCC |
| Archaea-16S | S-DArch-0349-a-S-17 | (CT)-GYGCASCAGKCGMGAAW |
| (Klindworth et al., 2013) | S-DArch-0519-a-A-16 | (CC)-TTACCGCGGCKGCTG |
| Protozoa-18S | Syl316-F | (GC)-GTCTTCGWTGGTAGTGTATT |
| (Sylvester et al., 2004) | Syl539-R | (CT)-CTTGCCCTCYAATCGTWCT |

Amplicons were purified and concentrated to a final 30 µL volume using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). They were then loaded onto a 2% agarose gel into three separate pools (3 × 10 µL). Bands were visualized, excised under ultraviolet radiation, and gel purified with the GENECLEAN Turbo kit (Qiagen) according to manufacturer instruction. Concentration of gel-purified amplicons loaded onto a 2% agarose gel was estimated using a low DNA mass Ladder (Invitrogen Corporation, Carlsbad, CA, USA), and an imaging system Chemimager (Alpha Innotech, San Leandro, CA, USA).

### *1.2. Construction of the library and sequencing steps (Figure 1)*

The final library was constructed by pooling samples with a mixing ratio of 8:1:1 for bacteria, protozoa and archaea, respectively (Kittelmann et al., 2013). The library was loaded for one Nanorun on a MiSeq sequencer (Illumina). Analysis steps consisted in (Illumina, 2010):

1/ Hybridization on flow cell: Double stranded DNA or cDNA were denaturated and single stranded fragments attached to the inside surface of a flow cell.

2/ Bridge amplification: This pre-sequencing amplification step allowed creation of millions of single stranded copies from template DNA or cDNA.

3/ Sequencing-by-synthesis: Each sequencing cycle consisted in i) addition of the four different labeled nucleotides and a DNA polymerase; ii) ligation of the labeled nucleotides to the first base of a single stranded fragment thanks to the enzyme; iii) laser excitation, lecture of the fluorescence emitted for each ligated nucleotide, identification of the first base of the fragment, knowing that fluorescence was different between nucleotides; iv) washout of non-used nucleotides. Each cycle added one nucleotide to each single stranded fragment. Sequencing cycles were 250-times repeated to get a minimum of 500 000 single stranded sequences (reads). The minimum number of single reads per sample was then calculated as the ratio between the minimum number of single reads generated during the run, out of the number of samples in the original library.

This approach was recently developed in the laboratory. In this experiment, we faced some difficulties during library preparation and sequencing. Thought several optimizations (new design of sequencing primer, PCR optimization) were made, we never obtained good sequencing yield. This approach gave good results with DNA libraries from other projects, which suggest that the reverse transcription of RNA to cDNA may be a step introducing biases in further sample manipulation. Currently, work is under progress to assess, by an

automated method (fluidigm amplification followed by MiSeq sequencing) the diversity of bacteria, archaea and protozoa in the same rumen samples, by targeting genes coding for 16S and 18S rRNA from genomic DNA.

### *1.3. Advantages and disadvantages of the technique (Di Bella et al., 2013; Kozich et al., 2013)*

In terms of samples preparation, multiplex sequencing is a cost-effective method, which allowed simultaneous processing of a large number of samples in a single run. Concerning sequencing, until recently, the Roche 454-sequencing technique was widely applied to assess rumen microbiota diversity. This expensive technique provides a small number of long reads (until 700 bp) allowing a high precision for species identification. Inversely, the MiSeq technology as used in this thesis gives the largest number of sequences per euro, which allowed covering a larger diversity of microbiota. However, compared to 454-sequencing, species identification is less precise, due to the shorter reads length.

## II. ANNEX 2 - CALCULATIONS USED TO ESTIMATE *IN VIVO* RUMINAL HYDROGEN PRODUCTION AND CONSUMPTION

### *2.1. Hydrogen consumption during methane production*

Quantities of $H_2$ (moles/day) consumed in the rumen for methanogenesis ($H2_{utilCH4}$) were estimated knowing that 4 moles $H_2$ are required to produce 1 mole $CH_4$:

$$H2_{utilCH4} = (m_{CH4}/M_{CH4}) \times 4$$

Where $m_{CH4}$: daily $CH_4$ production (g/day); $M_{CH4}$ = molecular weight of $CH_4$ (16 g/mol).

### *2.2. Hydrogen production and consumption during VFA synthesis*

Quantities of $H_2$ produced and consumed during VFA synthesis were calculated from observed rumen VFA profile and total VFA production estimated from the rumen fermentable organic matter content in diets (Nozière et al., 2010).

2.2.1. Calculation of rumen fermentable organic matter in diets

Rumen fermentable organic matter (MOF, g/kg DM) was calculated according to the equation 42 in Sauvant and Nozière, 2013:

$$MOF = MOD – (PDIA + AMDint + NDFDint + AGDint + PF)$$

Where MOD: digestible organic matter in diet (g/kg DM); PDIA: protein digestible in the intestine (g/kg DM); AMDint: starch digestible in the intestine (g/kg DM); NDFDint: NDF digestible in the intestine (g/kg DM); AGDint: fatty acid digestible in the intestine (g/kg DM); PF: products from silage fermentation. MOD was calculated by multiplying the organic matter content of the diets (OM, g/kg DM) by *in vivo* measurement of total tract organic matter digestibility (dOM, %). PDIA was estimated from diets composition and from the levels of PDIA in the individual ingredients given by INRA tables (INRA, 2010). AMDint was estimated from the level of starch reaching the duodenum (equation 31; Sauvant and Nozière, 2013), which was estimated by subtracting the theoretical amount of degraded starch in the rumen (equation 13; Sauvant and Nozière, 2013) to the total starch content of the diets. NDFDint was estimated from dOM (equations 33, 34 and 35; Sauvant and Nozière, 2013). AGDint was estimated from the fatty acid content of the diets (equations 36 and 37; Sauvant and Nozière, 2013). PF was estimated from INRA tables (INRA, 2010) and from the percentage of silage in diets.

2.2.2. Calculation of total and individual VFA production

Total VFA produced ($tVFA_{prod}$, moles/day) were calculated according to Nozière et al., 2010:

$$tVFA_{prod} = [(8.36 - 1.1 \times (PCO - 0.43)) \times MOF/1000] \times DMI$$

Where PCO: percentage of concentrate in the diets; DMI: daily DM intake (kg/day). From $tVFA_{prod}$, individual VFA productions ($Cx_{prod}$, moles/day) were calculated with the observed *in vivo* VFA profile in the rumen:

$$C2_{prod} = tVFA_{prod} \times C2 \text{ proportion in the rumen}$$
$$C3_{prod} = tVFA_{prod} \times C3 \text{ proportion in the rumen}$$
$$C4_{prod} = tVFA_{prod} \times C4 \text{ proportion in the rumen}$$
$$C5_{prod} = tVFA_{prod} \times C5 \text{ proportion in the rumen}$$

2.2.3. Calculation of hydrogen production and consumption during VFA synthesis

The amount of $H_2$ produced during VFA synthesis ($H2_{prodVFA}$, moles/day) was finally calculated knowing that 2 moles $H_2$ are generated per mole C2 or C4 produced:

$$H2_{prodVFA} = 2 \times C2_{prod} + 2 \times C4_{prod}$$

The quantities of $H_2$ consumed during VFA synthesis ($H2_{utilVFA}$, moles/day) were calculated knowing that 1 mole $H_2$ is required to produce 1 mole C3 or C5:

$$H2_{utilVFA} = 1 \times C3_{prod} + 1 \times C5_{prod}$$

## *2.3. Hydrogen production and consumption during microbial biomass synthesis*

Microbes growing on amino acids would produce 0.58 moles $H_2$ per kg dry microbial matter whereas microbes growing on NPN would consume 0.41 moles $H_2$ per kg dry microbial matter (Mills et al., 2001). Then, to calculate the amount of $H_2$ produced and consumed by microbes in our experiments, we first estimated the production of dry microbial matter from calculated microbial proteins production in the rumen (MAMIC, kg/day; equation 47, Sauvant and Nozière, 2013):

$$MAMIC = (40.7 + 75.6 \times 10^{-3} \times MOF + 8.07 \times PCO) \times DMI$$

Where MOF, PCO and DMI were as previously defined.

The production of microbial organic matter (MOM, kg/day) was then calculated knowing that the factor of conversion between microbial protein and nitrogen content is 6.25, and that 100 g MOM is made of ~9 g N (lab database):

$$MOM = (MAMIC/6.25) \times (100/9)$$

The production of microbial dry matter (MSM, kg/day) was finally estimated knowing that 100 g microbial dry matter would be made of 87.1 g microbial organic matter (Dijkstra et al., 1992):

$$MSM = ((MOM \times 100)/87.1))/1000$$

We estimated that 70% of N supplied in diets of our experiments came from amino acids, the rest coming from NPN. Then, production of $H_2$ from microbes growing on amino acids ($H2_{prodMIC}$, moles/day) was estimated as follow:

$$H2_{prodMIC} = MSM \times 0.58 \times 0.70$$

Quantities of $H_2$ consumed by microbes growing on NPN ($H2_{utilMIC}$, moles/day) were calculated as follow:

$$H2_{utilMIC} = MSM \times 0.41 \times 0.30$$

### *2.4. Hydrogen consumption during nitrate reduction and lipids biohydrogenation*

Quantities of $H_2$ consumed during nitrate reduction ($H2_{utilNO3}$, moles/day) or lipids biohydrogenation ($H2_{utilFA}$, moles/day) were estimated for diets including nitrate or lipids, knowing that the reduction of one mole nitrate to one mole ammonia requires 4 moles $H_2$ and that biohydrogenation of 1 mole C18:1, C18:2 or C18:3 requires 1, 2 or 3 moles $H_2$:

$$H2_{utilNO3} = (m_{NO3}/M_{NO3}) \times 4$$

$$H2_{utilFA} = (m_{C18:1}/M_{C18:1}) + 2 \times (m_{C18:2}/M_{C18:2}) + 3 \times (m_{C18:3}/M_{C18:3})$$

Where $m_{NO3}$: added nitrate (g/day); $M_{NO3}$: molecular weight of nitrate (62 g/mol); $m_{C18:1}$: added C18:1 (g/day); $M_{C18:1}$: molecular weight of C18:1 (282.5 g/mol); $m_{C18:2}$: added C18:2 (g/day); $M_{C18:2}$: molecular weight of C18:2 (280.5 g/mol); $m_{C18:3}$: added C18:3 (g/day); $M_{C18:3}$: molecular weight of C18:3 (278.5 g/mol).

### *2.5. Estimated quantities of produced and consumed hydrogen in the three in vivo experiments of this thesis*

Details of estimated quantities of produced and consumed $H_2$ in the three *in vivo* experiments of this thesis are presented in Table 2.

**Table 2** Estimated quantities of produced and consumed hydrogen in the four *in vivo* experiments of this thesis testing methane-mitigating strategies having different effects on the rumen hydrogen pool

| Experiment | Production of $H_2$ (moles/day) | | | Consumption of $H_2$ (moles/day) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | From C2 and C4 synthesis | From microbes synthesis | Total | For $CH_4$ synthesis | For C3 and C5 synthesis | For microbes synthesis | For nitrate reduction | For lipids biohydrogenation | Total |
| Experiment 1 (N.L. cows) | | | | | | | | | |
| CON | 83.9 | 0.85 | 84.7 | 77.2 | 8.8 | 0.26 | 0.0 | 0.00 | 86.3 |
| LIN | 80.6 | 0.86 | 81.5 | 63.2 | 10.8 | 0.26 | 0.0 | 1.55 | 75.8 |
| NIT | 85.9 | 0.85 | 86.8 | 59.5 | 8.3 | 0.26 | 18.3 | 0.00 | 86.3 |
| LIN+NIT | 84.3 | 0.85 | 85.2 | 51.7 | 8.5 | 0.26 | 18.3 | 0.14 | 78.8 |
| Experiment 2 (L. cows) | | | | | | | | | |
| CON | 113.2 | 1.35 | 114.6 | 116.2 | 21.4 | 0.41 | 0.0 | 0.00 | 138.0 |
| LIN+NIT | 103.0 | 1.15 | 104.2 | 62.7 | 13.9 | 0.35 | 21.0 | 3.85 | 101.7 |
| Experiment 3 (N.L. cows) | | | | | | | | | |
| CON | 80.0 | 0.84 | 80.9 | 78.1 | 9.1 | 0.25 | 0.0 | 0.00 | 87.4 |
| TEA | 80.9 | 0.83 | 81.7 | 73.5 | 8.9 | 0.25 | 0.0 | 0.00 | 82.6 |
| NIT | 83.3 | 0.83 | 84.1 | 54.8 | 7.9 | 0.25 | 17.8 | 0.00 | 80.7 |
| TEA+NIT | 79.8 | 0.81 | 80.6 | 51.6 | 8.6 | 0.25 | 17.5 | 0.00 | 77.9 |
| Experiment 3 (L. cows) | | | | | | | | | |
| CON | 105.3 | 1.27 | 106.6 | 108.8 | 18.6 | 0.39 | 0.0 | 0.00 | 127.7 |
| TEA | 99.9 | 1.14 | 101.1 | 110.6 | 14.5 | 0.34 | 0.0 | 0.00 | 125.4 |

N.L. cows: non-lactating cows; L. cows: lactating cows.

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